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REMARKS/ARGUMENTS

In response to the Advisory Action mailed April 2, 2004, and further to the Request For Continued Examination filed herewith, Applicants request further consideration and examination in view of the above amendments and following remarks.

Claim 19 has been added. Claims 1, 4, 6-8, 10-14 and 19 are pending in this application.

The Prior Art Rejection

The sole issue remaining in this application is whether the claims (1, 4, 6-8, 10-14 and 19) are patentable under 35 U.S.C. §103(a) over JP 9-117279 in view of JP 1-304882. More specifically, the issue is whether it would have been obvious to one having ordinary skill in the art at the time the invention was made to utilize sucrose as a stabilizing agent for lecithin-modified superoxide dismutase (such as disclosed in the JP '279 reference) in view of the teachings of the JP '882 reference which discloses that human SOD can be stably stored in a freeze-dried state by mixing the SOD with "at least one of disaccharides, monosaccharides such as ketose or sugar alcohol."

The Examiner has taken the position that lecithin-modified superoxide dismutase (hereinafter abbreviated "PC-SOD") "possesses more similarities to SOD than differences, and that the similarities are much more relevant to stabilization or storage concerns than the differences." The Examiner has claimed that his position is based on "the fact that both the derivatized enzyme and its non-derivatized counterpart catalyze the same chemical reaction, and therefore have the same therapeutic utility." From this alleged "fact," the Examiner has speculated that "at the very least the active site of the enzymes are the same," and therefore, "one of ordinary skill in the art would have reasonably expected that a compound, i.e., sucrose, which preserved the active site of the non-derivatized enzyme, would have also preserved the active site of the derivatized enzyme."

Contrary to the Examiner's statement, there is not any evidence of record that establishes as a fact that PC-SOD and SOD "have the same therapeutic utility." None of the references of record actually teach or suggest that PC-SOD and SOD have "the same

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therapeutic utility.” Further, Applicants have not made any statements on the record that would suggest that PC-SOD and SOD “have the same therapeutic utility.” Thus, the underlying “fact” upon which the rejection is based is nothing more than an unsupported premise. Further, as discussed below, Applicants have submitted herewith publications that support a factual finding that PC-SOD and SOD have different therapeutic utility.

Further, the claimed invention is directed to a composition containing PC-SOD that has been lyophilized and stabilized with sucrose to prevent degradation of the lecithin moieties during long term storage, and to facilitate rapid reconstitution (re-dissolution) of the PC-SOD. Obviously, these objectives do not relate to preservation or stabilization of the active site of the SOD moiety. Thus, speculation regarding the preservation of the active site of SOD is not particularly relevant to the problems solved by the claimed invention.

Chemical And Biological Differences Between PC-SOD And SOD

PC-SOD is prepared by bonding to human SOD one or more (four on average) lecithin derivatives in which a chemical cross-linking agent is bonded to the hydroxyl group at the 2nd position of lysolecithin, and it has been patented in the United States (USP-5109118). SOD is water-soluble. However, PC-SOD is more hydrophobic because lecithin is bounded to SOD. In this point, they have completely different chemical and biological properties.

Document A (*The Journal of Pharmacology and Experimental Therapeutics*, Vol. 262, No. 3, p. 1214-1219 (1992)) describes that lecithinized SOD (PC-SOD) has a Forssman antiserum-induced effect. Document A also describes that PC-SOD delays its disappearance in plasma while retaining SOD activity, and PC-SOD has 4 to 20 times higher cell affinity and 200 times or more pharmacological potency than SOD. It is thus concluded that PC-SOD is applicable to various clinical uses. Further, Document B (the above-mentioned *Journal*, Vol. 271, No. 3, p. 1672-1677 (1994)) describes that PC-SOD enhances pharmacologic activity by increasing cell membrane affinity; PC-SOD exerts 100-fold potency than SOD in vascular endothelial cell damage caused by active oxygen; and PC-SOD exerts an effect on ischemia-reperfusion paw edema. However, it is described therein that SOD and PEG-SOD do not have such effect.

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As described above, PC-SOD remarkably differs from SOD in chemical and biological properties and therefore it cannot be said that the difference therebetween is only in that an active site is preserved.

Sorbitol Applicability

In the specification of the present application, Table 5 of Example 1 shows enzyme activity during a long storage. A lyophilized product containing sucrose (prescription 1) and a lyophilized product containing sorbitol and mannitol (prescription 3) had almost the same results. Enzyme activity tests generally show large variations. The prescription 3 increased its activity to 114 after 3-month storage at 40°C from 100 obtained immediately after lyophilization. This was attributable to a variation. Thus, the results described therein should be considered data indicating that no loss of enzyme activity is under all the conditions. On the other hand, Table 7 shows the detection test results of low-molecular weight analogues (degradation products) by reversed phase chromatography (220 nm). The lyophilized product containing sucrose (prescription 1) was compared with the lyophilized product containing sorbitol and mannitol (prescription 3). The prescription 3 had 1.5 times, 3.4 times, 2.2 times, and 1.6 times more amount of detected analogues than the prescription 1 immediately after lyophilization, after 3-month storage at 40°C, 3-month storage at 25°C, and 3(6)-month storage at 8°C, respectively. These prove that the blending of sorbitol and mannitol allows low-molecular weight degradation products to be easily formed. It can be thus concluded that sorbitol also is not preferable for stabilization. Accordingly to JP 1-304882, dimmers are generated as byproducts by lack of SOD stability. It is described therein that the purpose of SOD stabilization can be achieved by any of disaccharides, monosaccharides such as ketoses, and sugar alcohols. Due to this point, the present invention can be said to largely differ from JP 1-304882 in that the purpose of the present invention cannot be achieved by any saccharide other than sucrose, meaning that they are different in dissolution process and decomposition products to be formed.

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Reason Why Sucrose Was Selected

Sorbitol is not preferable for stabilizing PC-SOD. On top of that, the shape of a composition cake is unfavorably turned into paste in terms of properties after lyophilization. When ultra pure water is added thereto, the dissolution becomes a time-consuming process, and a little amount of insoluble foreign substance was observed (see Table 2). These facts are considered significant drawbacks when PC-SOD is available as a vial product. Consequently, sorbitol cannot be used in terms of product development.

Further, PC-SOD is prepared by bonding to protein (SOD) a surface active compound (lecithin), and it easily turns into paste unlike a substance composed of only protein in lyophilization process. Hence, this property of a lyophilized product has been a problem. This present invention is significant in that this problem can be solved with sucrose rather than sorbitol.

Commercial Success

PC-SOD is currently being developed as an agent for improving doxorubicin-induced cardiotoxicity (Document C: *Toxicology and Applied Pharmacology* 194 (2004) 180-188). This development promotes the use of doxorubicin, an anticancer agent, and will make a great social contribution. Thus, it can be said that the drug composition of the present invention has medical importance.

In view of the foregoing, the present invention stabilizes a lyophilized product for a long term by blending sucrose with PC-SOD and provides the product with excellent properties, which cannot be accomplished by any stabilizer other than sucrose. Therefore, the present invention cannot be easily made based on JP 1-304882.

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CONCLUSION

In view of the above amendments and remarks, it is respectfully submitted that the application is in condition for allowance and notice of the same is earnestly solicited.

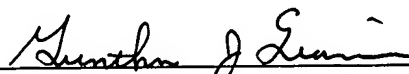
Respectfully submitted,

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June 4, 2004

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Attachments

Lecithinization of Superoxide Dismutase Potentiates Its Protective Effect against Forssman Antiserum-Induced Elevation in Guinea Pig Airway Resistance

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ABSTRACT

We synthesized lecithinized superoxide dismutase (PC-SOD), in which a lecithin derivative was covalently bound to recombinant human SOD. We selected PC4-SOD (4 molecules of the lecithin derivative bound to each SOD dimer) and PC10-SOD (10 molecules of the derivative to each SOD dimer) for our study. Both of these PC-SOD retained SOD activity *in vitro* and showed delayed plasma disappearance *in vivo* in rats. PC-SOD also had 4 to 20 times higher affinity *in vitro* for several kinds of cells than that of unmodified SOD. Because Forssman antiserum is known to induce bronchial obstruction in guinea pigs *via* actions of O_2^- ,

we studied the effect of PC-SOD on Forssman antiserum-induced respiratory resistance. Unmodified SOD was ineffective at the doses of 1,000 to 30,000 U/kg, whereas PC-SOD showed a dose-dependent inhibitory effect over the range of 10 to 1,000 U/kg. The ED_{50} of PC4-SOD and PC10-SOD were 140 and 240 U/kg, respectively, at 30 min after the challenge with Forssman antiserum. These findings suggest that the pharmacological potency of PC-SOD is over 200 times more than that of unmodified SOD, and that PC-SOD may have the potential for various clinical applications.

O_2^- acts as an exacerbating factor in various disease states, such as allergic diseases (Brigham, 1986), myocardial ischemia (Gardner *et al.*, 1983) and Crohn's disease (Suematsu *et al.*, 1987). SOD, an enzyme that scavenges O_2^- , has been suggested as a possible therapeutic tool for treating those diseases. However, its rapid metabolic clearance and low affinity to cell membranes have been found to limit its clinical application. One way to overcome such disadvantages is to incorporate SOD into a drug delivery system. Several chemically modified forms of SOD, including SOD covalently bound to polyethylene glycol (Veronese *et al.*, 1983; Miyata *et al.*, 1988) and pyran-SOD, which utilizes a pyran copolymer (Oda *et al.*, 1989), have not yet been approved for clinical use because of their insufficient pharmacological potency.

We have developed a drug delivery system in which lipid microspheres consisting of soybean oil surrounded by PC are used to carry several drugs to their targets. Some investigators, including ourselves, have found that PC is highly cytotoxic

and safe (Mizushima *et al.*, 1987; Mizushima, 1991). We found that the affinity of immunoglobulin G to some cells and tissues increased markedly when immunoglobulin G was covalently bound to PC (Mizushima and Igarashi, 1991).

In order to develop a more effective drug delivery system for SOD, we planned to synthesize PC-SOD, which had an amide bond (covalent bond) between a PC derivative and SOD. We synthesized an active ester form of a PC derivative and bound the ester covalently to SOD to produce PC-SOD. The cellular affinity, tissue distribution and plasma disappearance of the PC-SOD were then determined. The pharmacological activity of PC-SOD was evaluated by its inhibitory effect on the increase in respiratory resistance induced by Forssman antiserum in guinea pigs.

Materials and Methods

rhSOD

rhSOD (Asahi Chemical Ind. Co., Ltd., Shizuoka, Japan) was used in this study. In this preparation, cysteine at position 111 has been replaced by serine to enhance the chemical stability of the parent

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ABBREVIATIONS: SOD, superoxide dismutase; rhSOD, recombinant human CuZn-SOD; PC, lecithin, phosphatidylcholine; C_3 PC, 2-(4-hydroxycarbonylbutyryl) lysolecithin; PC-SOD, lecithinized SOD, rhSOD covalently bound to C_3 PC; $T_{1/2} \alpha$, half-time of plasma disappearance in distributive phase; $T_{1/2} \beta$, half-time of plasma disappearance in final phase; MCR, metabolic clearance rate; O_2^- , superoxide anion; BSA, bovine serum albumin; DMF, dimethyl formamide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute.

compound. Each molecule is a dimer of about 32,000 Da and contains 22 lysines and 2 terminal α -amino groups. The isoelectric point is 5.2.

Animals and Cancer Cells

Six-week-old male C₃H/HeNCrj mice and 7-week-old male Wistar rats were purchased from Nihon Seibutsu Zairyo (Tokyo, Japan). Female Hartley guinea pigs weighing 300 to 400 g were purchased from Nihon Ikagaku Dobutsu Shizai Kenkyusho Inc. (Tokyo, Japan). Murine sarcoma 180, MM46 cancer cells from Teijin Co., Ltd. (Tokyo, Japan) and L1210 cancer cells from Cancer Institute (Japanese Foundation for Cancer Research, Tokyo, Japan) were kindly provided.

Reagents

Forssman antiserum (an anti-sheep erythrocyte antibody) and *o*-phenylenediamine were purchased from Organon Teknica N.V. Cappel Products (Durham, NC), and peroxidase-labeled anti-mouse immunoglobulin came from Tago Inc. (Burlingame, CA). Lysolecithin, amidol, perchloric acid, cytochrome C (bovine heart type V), xanthine oxidase (EC. 1.1.3.22, 0.25 U/ml), xanthine, BSA and N, N-dicyclohexyl carbodiimide were all obtained from Sigma Chemical Co. (St. Louis, MO), Ficoll Paque came from Pharmacia (Uppsala, Sweden), RPMI 1640 medium was from Gibco (Grand Island, NY) and Hionic Fluor scintillation cocktail and Soluene 350 solubilizer were from Packard Company Inc. (Meriden, CT). Fetal bovine serum (No. 33510, lot 1-1038) was obtained from Men-eki Seibutsu Kenkyusho (Gunma, Japan), and N-succinimidyl [2,3 ³H]-propionate (0.021 mol/37.0 MBq) came from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Other general reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-SOD antibodies (rabbit and mouse) were given by Toxicology Research Laboratory, Asahi Chemical Ind. Co., Ltd. (Shizuoka, Japan).

Synthesis of PC-SOD

C₃PC. Lysolecithin (2 g, 3.92 mmol) was dissolved in chloroform/pyridine (80 ml/20 ml) and then glutaric anhydride (1.1 g, 9.81 mmol) and 4-dimethylaminopyridine (1.2 g, 9.81 mmol) were added. The mixture was stirred at 50°C for 12 h under nitrogen atmosphere and the solvent was evaporated under reduced pressure. The residue was dissolved in a mixture of chloroform, methanol and water (4:5:1), applied to a column [Dowex 50w-X8 (10 × 10 cm)] and eluted with the same solvent system. The fraction containing the target substance, C₃PC, was then collected and evaporated under reduced pressure, and the residue was purified using an open octadecylsilane column [the mobile phase was a mixture of acetone and 0.5% pyridine in aqueous solution (45:55)]. This process gave 1.9 g of C₃PC (3.14 mmol, yield 80%).

The rate of flow value of C₃PC was 0.1 when thin-layer chromatography was performed with a mobile phase of chloroform, methanol and water (65:25:4). Proton nuclear magnetic resonance spectroscopy of C₃PC in CDCl₃ is as follows (JEOL 270FX): δ : 0.84 (t, 3H), 1.20 (brs), 1.52–1.60 (brs, 2H), 1.80–1.95 (m, 2H), 2.20–2.24 (m, 6H), 3.35 (s, 9H), 3.78 (m, 4H), 3.90–4.35 (m, 4H) and 5.20 (m, 1H).

Activation of C₃PC. Synthesis of activated C₃PC and PC-SOD was done by the method of Anderson *et al.* (1964), with a modification. The C₃PC (1.9 g, 3.14 mmol) obtained in the above experiment was dissolved in 58 ml of methylene chloride and cooled to 0°C. Dicyclohexylcarbodiimide (810 mg, 3.93 mmol) dissolved in a mixture of N-hydroxysuccinimide (360 mg, 3.93 mmol) and tetrazole (220 mg, 3.14 mmol) was added to this solution. The solution was then stirred at 25°C for 12 h. The resulting precipitate was removed using cerite filtration, and the solvent of the filtrate was evaporated under reduced pressure. The residue was then dissolved in 60 ml of DMF to serve as the activated C₃PC solution (0.052 mmol/ml DMF). The R_f value of the activated C₃PC was 0.4 on thin-layer chromatography with a mobile phase of chloroform/methanol/water (65:25:4). The chemical structure of activated C₃PC is shown in figure 1.

Binding of C₃PC to SOD. To 40 ml of SOD [400 mg (0.0125 mmol) in 50 mM borate buffer, pH 8.5] was added 8 ml of DMF, and the

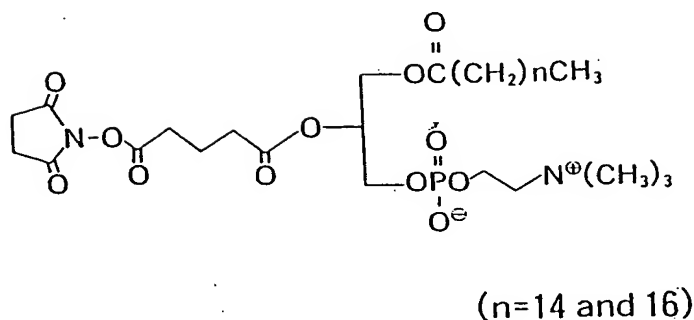


Fig. 1. Activated C₃PC.

solution was cooled to 0°C. Activated C₃PC (0.12 mmol in 32 ml of DMF) was added to the cooled solution over 5 h, and the solution was then stirred at 0°C for a further 12 h. The resulting solution was purified using ion-exchange column chromatography with Q-Sepharose FF (Pharmacia Inc.) and isoelectrofocusing was used to identify the target substance. The method of Lowry was used to measure the protein content, and this was then used to calculate the concentration of SOD (Lowry *et al.*, 1951). *In vitro* SOD activity of PC-SOD was measured using the xanthine-xanthine oxidase method (McCord and Fridovich, 1969). The number of C₃PC molecules bound to each SOD molecule was determined using the TNBS method, by which free amino groups can be quantitatively analyzed (Fields *et al.*, 1967). This synthetic process afforded PC-SOD having four molecules of C₃PC per SOD dimer (PC4-SOD). When 0.60 mmol of activated C₃PC was used, PC-SOD having 10 molecules of C₃PC per SOD dimer was obtained (PC10-SOD).

Isoelectrofocusing

The isoelectric point of PC-SOD was determined according to the method of Awdeh *et al.* (1968). An electrophoresis apparatus (Multiphor II, Pharmacia Inc.) was used. Electrophoresis conditions were as follows: The electrode solution was 0.1 M glutamic acid in 0.5 M phosphoric acid at the anode and 0.1 M β -alanine at the cathode. The fixing solution was 57.5 g of trichloroacetic acid and 17.25 g of sulfosalicylic acid dissolved in distilled water to make 500 ml. For the bleaching solution, a mixture of 500 ml of ethanol and 160 ml of acetic acid was mixed with distilled water to make up 2000 ml. The dyeing solution was 0.46 g of Coomassie blue R250 dissolved in 400 ml of the bleaching solution, and the protective solution was 40 ml of glycerine plus 360 ml of the bleaching solution.

³H-Labeling of PC-SOD

Synthesis was performed according to the method of Kummer *et al.* (1981). To 37 MBq of [³H]N-succinimidyl propionate with the solvent evaporated, either unmodified SOD, PC4-SOD or PC10-SOD was added (1 mg SOD/ml 0.01 M PBS, pH 7.2). The resultant solutions were first stirred in an ice water bath for 30 min and then at room temperature for 3 h. The solutions were then dialyzed in PBS to obtain [³H]unmodified SOD (71.0 kBq/10 μ g), [³H]PC4-SOD (61.4 kBq/10 μ g) and [³H]PC10-SOD (35.5 kBq/10 μ g), respectively.

Measurement of the Cellular Affinity of PC-SOD

Heparinized peripheral blood was obtained from healthy adult human volunteers. Lymphocytes and neutrophils were separated using conventional methods (Lippman and Barr, 1977; Peterson *et al.*, 1976) and were suspended in RPMI 1640 medium with 10% fetal bovine serum at 2×10^6 cells/ml. To 1-ml aliquots of this suspension, [³H]unmodified SOD, [³H]PC4-SOD or [³H]PC10-SOD was added in an amount equivalent to 5 μ g of SOD. After incubation for 3 h at 37°C in a 5% CO₂ atmosphere, each solution was rinsed five times with 0.01 M PBS (pH 7.4) containing 0.005% Tween 20. The radioactivity of the [³H]unmodified SOD, [³H]PC4-SOD or [³H]PC10-SOD bound to the

white blood cells was then measured, and its percentage of the total ^3H added was calculated. The obtained percentage included both intracellular SOD and SOD bound to the cell surface, and was used as an index of cellular affinity.

Murine sarcoma 180, MM46 and L1210 cancer cells were grown by i.p. inoculation and suspended in RPMI 1640 medium with 10% fetal bovine serum at 5×10^6 cells/ml. The affinity of PC-SOD for these cells was measured in the same way as for human blood cells.

Plasma Concentration of PC-SOD after i.v. Injection in Rats

Blood was collected from the carotid arteries of 7-week-old male Wistar rats at various times after the administration of PC-SOD or unmodified SOD into the tail vein at a SOD equivalent dose of 1 mg/kg. To make a sample solution, plasma was separated and diluted 10-fold with 0.1% BSA in 0.01 M PBS, pH 7.4. Then, the SOD concentration of the sample solution was measured by a sandwich enzyme-linked immunosorbent assay (Kasahara *et al.*, 1987). The washing solution (0.2% Tween 20 saline), and the diluent (0.01 M PBS, pH 7.4, containing 0.1% BSA) were used. The calibration curve was drawn with known concentrations of a PC-SOD solution which had been treated in the same way as the sample solution. Least squares linear regression analysis was used for calculation of the half-time of plasma disappearance (distributive phase, $T_{1/2} \alpha$; final phase, $T_{1/2} \beta$) and the MCR using a two-compartment model analysis (Tait *et al.*, 1961).

Tissue Distribution of PC-SOD in Rats

Seven-week-old male Wistar rats were administered either PC-SOD or unmodified SOD *via* the tail vein (at a dose equivalent to 1 mg/kg SOD). After 2 h, the rats were exsanguinated and then perfused with saline *via* the aorta. The brain, lungs, heart, spleen, liver and kidneys were removed and homogenized with a 5-fold volume of 0.1% BSA/PBS. After centrifugation at 3000 rpm, the supernatant was stored at -80°C until analysis. The SOD concentration in each tissue was measured in the same way as that in serum using the sandwich enzyme-linked immunosorbent assay mentioned above.

Measurement of Respiratory Resistance in Guinea Pigs

Respiratory resistance of guinea pigs was measured by modifying the oscillation method of Mead (1960). A type PMR-2 apparatus (Shizume Medical Co., Ltd., Tokyo, Japan) was used to measure the respiratory resistance in guinea pigs, unanesthetized and breathing spontaneously. PC-SOD was injected through the ear vein and, 5 min later, Forssman antiserum diluted 40-fold with saline was given i.v. at a dose of 1 ml/kg. The respiratory resistance was measured at 0.5, 1, 2, 5, 10, 15, 20, 25 and 30 min after the challenge, and the data at 0.5, 1, 2, 10, 20 and 30 min are shown in table 3. The control animals were administered saline instead of sample solution. The values of drug required to produce 50% of maximal effect (ED_{50}) were obtained using Probit's method.

The animal studies in this report were approved by the Animal Experimentation Committee of St. Marianna University.

Results

TNBS method and isoelectrofocusing. The TNBS method confirmed PC4-SOD and PC10-SOD to have 4 and 10 molecules (on average) of C_3PC covalently bound to each SOD dimer, respectively. The results of isoelectrofocusing of PC4-SOD and PC10-SOD are shown in figure 2. Both of the derivatives showed three to four bands and even unmodified SOD showed bands for isomers other than the principal one. The isoelectric point is 5.2 for unmodified SOD, approximately 4.2 for PC4-SOD and approximately 3.5 for PC10-SOD.

SOD activity. The *in vitro* activity of unmodified SOD by the xanthine-xanthine oxidase method was 3467 U/mg, whereas that of PC4-SOD was 2876 U/mg (equivalent to 83.0% of unmodified SOD) and that of PC10-SOD was 2000 U/mg (57.7%).

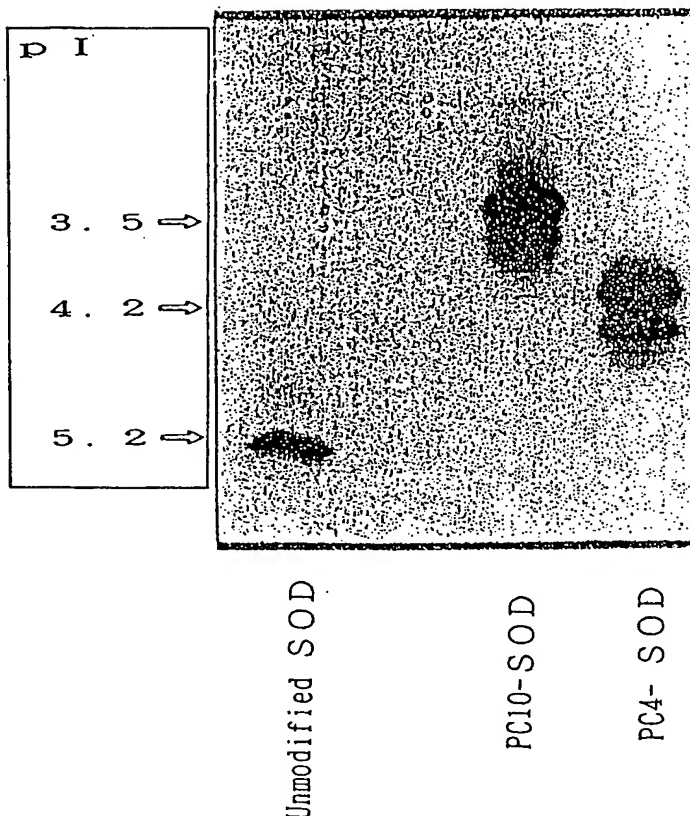


Fig. 2. Isoelectrofocusing of SOD preparations.

Cellular affinity. The amount of each PC-SOD bound to various cells is shown in table 1. Both PC4-SOD and PC10-SOD had a higher cellular affinity than unmodified SOD or a mixture of unmodified SOD and C_3PC (1 μg , equivalent to 5 μg of PC10-SOD as bound C_3PC).

Pharmacokinetic studies. Mean plasma disappearance curves for PC4-SOD, PC10-SOD and unmodified SOD after i.v. injection in rats are shown in figure 3. The mean $T_{1/2} \alpha$ values of unmodified SOD, PC4-SOD and PC10-SOD were 0.09, 0.14 and 0.14 h, respectively. The mean $T_{1/2} \beta$ values were 0.98 h for unmodified SOD, 1.67 h for PC4-SOD and 1.97 h for PC10-SOD. In addition, marked reduction of MCR of PC4-SOD (0.07 ml/h·kg) and PC10-SOD (0.04 ml/h·kg) were observed when compared with that of unmodified SOD (0.25 ml/h·kg).

Tissue distribution of PC4-SOD. Only the tissue distribution of PC4-SOD was studied because it demonstrated a stronger pharmacological potency than that of PC10-SOD in the respiratory resistance test.

The SOD concentrations in the brain, lungs, heart, liver and serum were all higher in the rats administered PC4-SOD than in those given unmodified SOD (table 2). The renal SOD concentration in the PC4-SOD group was lower than that in the unmodified SOD group.

Effects of PC-SOD on respiratory resistance. Intravenous injection of Forssman antiserum induced a transient sharp increase in respiratory resistance (the first phase) at 0.5 min, followed by a gradual increase of 40 to 50% in resistance (the second phase) at 2 to 30 min after injection. The respiratory

TABLE 1

Binding of PC-SOD and unmodified SOD to cells *in vitro*

Data are the mean \pm S.D. of bound [3 H]SOD to 10^6 cells, each value indicates $10^2 \times$ percentage of total [3 H]SOD added (5 μ g of SOD). Multiple Student's *t* test with the Bonferroni's correction was used for comparisons among groups.

| | <i>n</i> | Unmodified SOD | Mixture of SOD and C ₃ PC | PC-SOD | |
|--------------------------|----------|-----------------|--------------------------------------|------------------|------------------|
| | | | | PC4-SOD | PC10-SOD |
| Human lymphocytes | 6 | 0.34 \pm 0.16 | 0.30 \pm 0.16 | 3.24 \pm 1.54* | 3.45 \pm 1.69* |
| Human neutrophils | 6 | 0.18 \pm 0.04 | 0.17 \pm 0.04 | 2.33 \pm 1.21* | 3.70 \pm 1.84* |
| Human endothelial cells | 4 | 0.28 \pm 0.16 | 0.27 \pm 0.12 | 6.00 \pm 3.17 | 5.80 \pm 2.50 |
| L1210 cancer cells | 4 | 0.11 \pm 0.02 | 0.13 \pm 0.01 | 1.10 \pm 0.41* | 1.43 \pm 0.42* |
| Sarcoma 180 cancer cells | 4 | 0.13 \pm 0.06 | 0.16 \pm 0.06 | 1.98 \pm 1.49 | 1.68 \pm 0.41* |
| MM46 cancer cells | 4 | 0.31 \pm 0.03 | 0.30 \pm 0.03 | 1.32 \pm 0.38* | 2.23 \pm 0.22* |

* *P* < .05 (vs. unmodified SOD or mixture of SOD and 1 μ g of C₃PC).

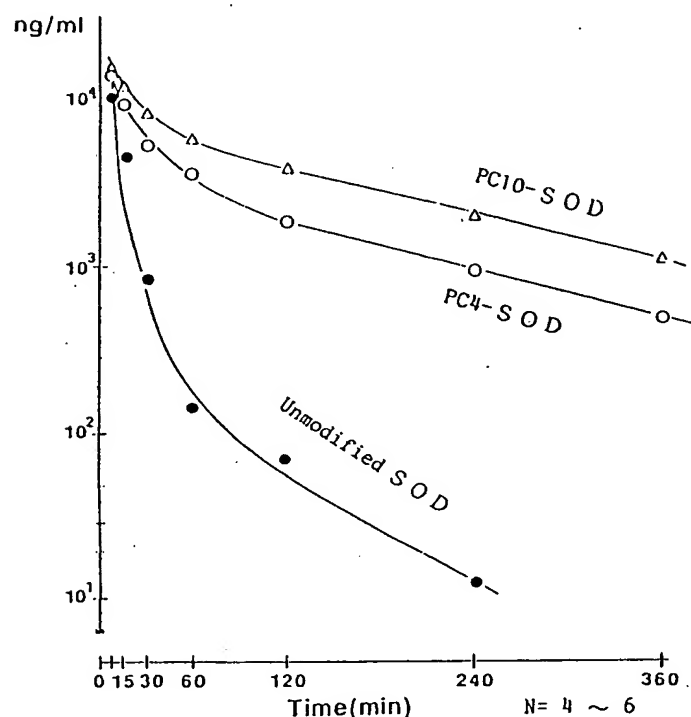


Fig. 3. Plasma SOD concentration after the i.v. injection of SOD preparations into rats.

TABLE 2

Tissue distribution of PC4-SOD in Wistar rats 2 h after i.v. injection

| | Unmodified SOD | PC4-SOD |
|---------------|------------------------|-------------------------|
| Brain (ng/g)* | N.D. | 14.0 \pm 0.8* |
| Lung (ng/g) | 18.1 \pm 4.0 | 242.8 \pm 64.1* |
| Heart (ng/g) | 11.7 \pm 2.7 | 253.2 \pm 14.6* |
| Liver (ng/g) | 19.3 \pm 1.9 | 255.5 \pm 24.7* |
| Kidney (ng/g) | 48,250.0 \pm 8,509.0 | 21,350.0 \pm 2,828.0* |
| Serum (ng/ml) | 14.2 \pm 2.4 | 2,733.3 \pm 232.0* |

* Total amount of SOD per 1 g of wet tissues. Data are described as mean \pm S.D. (*n* = 6).

* *P* < .005 vs. unmodified SOD (Student's *t* test with Bonferroni's correction).

resistance then gradually decreased and returned to the preinjection level after about 3 h.

Both PC4-SOD and PC10-SOD inhibited these changes in a dose-dependent manner (table 3). The ED₅₀ was 140 U/kg for PC4-SOD and 240 U/kg for PC10-SOD at 30 min after the injection of antiserum (table 4). On the other hand, unmodified

SOD showed no inhibitory effect within the dose range of 1,000 to 30,000 U/kg.

Neither a mixture of unmodified SOD (3,000 U/kg) and C₃PC (124 μ g/kg, equivalent to 3000 U of PC4-SOD as bound C₃PC) nor C₃PC only (124 μ g/kg) had any inhibitory effect (table 3). PC-SOD by itself did not affect the respiratory resistance in guinea pigs which had not been challenged with Forssman antiserum (data not shown).

Discussion

In the present study, we assessed the pharmacological activity of PC-SOD *in vivo* by Forssman antiserum-induced respiratory obstruction in guinea pigs. The Forssman antigen is abundant in guinea pigs (Tanaka and Leduc, 1956; Taighman *et al.*, 1972) and i.v. injection of Forssman antiserum therefore induces shock. This is classified as a type II allergic response (Redfern, 1926; Taighman *et al.*, 1972; Pelczarska and Roszkowski, 1973; Nagai *et al.*, 1980) and involves a biphasic change in airway resistance (Pelczarska and Roszkowski, 1973; Butler and Smith, 1981). Although the mechanism of this allergic reaction is not fully understood, recent studies have demonstrated that O₂⁻ is involved in the mechanism of the second phase of airway resistance or respiratory resistance (Nakagami *et al.*, 1983, 1991).

In the present study, we measured respiratory resistance to monitor the rate of airway obstruction because respiratory resistance can be measured in a noninvasive manner under normal respiration without using anesthesia. Both PC4-SOD and PC10-SOD inhibited the increase in respiratory resistance in a dose-dependent manner over the range of 10 to 1000 U/kg. On the other hand, unmodified SOD did not show any inhibitory effect even at a dose of 30,000 U/kg. These results suggest that the pharmacological potency of PC-SOD is over 200 times more than that of unmodified SOD.

Liposome-entrapped rhSOD (liposomal SOD) is reported to have an inhibitory effect on the second phase of the Forssman antiserum-induced increase in airway resistance at a dose of 30,000 U/kg (Nakagami *et al.*, 1991). Airway resistance and respiratory resistance cannot be compared directly because airway resistance is measured under anesthesia with artificial ventilation. However, it appears that PC-SOD exerts a pharmacological effect at a much lower dose than liposomal SOD. The pharmacological activity of liposomal SOD has been shown to depend on its affinity for cell membranes (Freeman *et al.*, 1983; Transwell and Freeman, 1987), and the clinical efficacy of liposomal SOD has already been demonstrated (Baillet *et al.*, 1986).

TABLE 3

Effect of PC-SOD on the respiratory resistance response to Forssman antiserum in guinea pigs.

Values are described as percent increase in respiratory resistance (mean \pm S.E.). Guinea pigs were injected i.v. with PC-SOD or unmodified SOD 5 min before the challenge with Forssman antiserum. Statistical analysis was treated by one-way analysis of variance followed by Dunnett's multiple group comparison test.

| Groups | | n | Time after Challenge | | | | | |
|---------------------------------------|-------------|----|----------------------|-------------|-------------|--------------|--------------|--------------|
| | | | 0.5 | 1 | 2 | 10 | 20 | 30 |
| min | | | | | | | | |
| Control PC4-SOD | Saline | 19 | 134.1 ± 14.3 | 34.9 ± 7.2 | 43.9 ± 5.7 | 43.4 ± 4.5 | 46.0 ± 4.6 | 43.7 ± 3.6 |
| | 10 U/kg | 6 | 119.8 ± 22.2 | 26.8 ± 7.7 | 39.5 ± 6.6 | 32.8 ± 9.6 | 49.5 ± 10.2 | 44.5 ± 7.9 |
| | 30 U/kg | 6 | 122.0 ± 26.0 | 33.0 ± 14.5 | 38.8 ± 6.1 | 35.3 ± 6.6 | 41.2 ± 6.1 | 34.7 ± 4.4 |
| | 100 U/kg | 7 | 84.2 ± 20.8 | 15.6 ± 6.9 | 22.9 ± 8.5 | 18.4 ± 7.7* | 20.1 ± 8.2* | 22.9 ± 7.9* |
| | 300 U/kg | 7 | 117.9 ± 14.5 | 18.0 ± 9.1 | 14.3 ± 6.1* | 11.1 ± 2.4** | 20.7 ± 3.9* | 11.4 ± 3.7** |
| PC10-SOD | 1,000 U/kg | 6 | 47.3 ± 10.4** | 13.8 ± 8.7 | 23.0 ± 13.3 | 5.8 ± 3.9** | 7.2 ± 4.5** | 7.8 ± 6.2** |
| | 10 U/kg | 6 | 153.0 ± 13.9 | 28.7 ± 9.0 | 42.7 ± 6.4 | 38.8 ± 2.4 | 41.2 ± 5.7 | 44.3 ± 10.8 |
| | 30 U/kg | 6 | 139.2 ± 33.2 | 25.5 ± 10.2 | 48.8 ± 14.1 | 40.2 ± 11.5 | 42.8 ± 11.2 | 37.0 ± 10.8 |
| | 100 U/kg | 7 | 169.6 ± 24.5 | 24.1 ± 10.0 | 19.7 ± 7.7 | 19.7 ± 5.6* | 25.1 ± 7.4 | 24.6 ± 7.8 |
| | 300 U/kg | 7 | 175.0 ± 16.6 | 27.4 ± 12.4 | 36.4 ± 8.3 | 23.1 ± 6.4 | 16.6 ± 5.8** | 15.0 ± 6.1** |
| Unmodified SOD | 1,000 U/kg | 7 | 122.5 ± 21.8 | 11.0 ± 5.5 | 17.1 ± 4.7* | 14.0 ± 5.6** | 13.7 ± 5.8** | 18.4 ± 7.7* |
| | 1,000 U/kg | 4 | 191.7 ± 79.1 | 20.8 ± 2.0 | 36.8 ± 15.6 | 42.0 ± 14.1 | 48.0 ± 11.8 | 40.0 ± 8.8 |
| | 3,000 U/kg | 7 | 109.6 ± 30.8 | 32.1 ± 9.3 | 54.1 ± 11.3 | 34.3 ± 7.9 | 38.9 ± 7.0 | 45.1 ± 8.7 |
| | 10,000 U/kg | 5 | 191.8 ± 42.9 | 15.3 ± 4.2 | 43.0 ± 2.6 | 28.8 ± 7.3 | 36.8 ± 5.7 | 42.6 ± 5.7 |
| | 30,000 U/kg | 5 | 152.8 ± 23.4 | 36.6 ± 22.6 | 41.4 ± 19.2 | 32.4 ± 13.7 | 42.6 ± 8.2 | 42.0 ± 5.4 |
| Unmodified SOD + C ₃ PC | 3,000 U/kg | 7 | 105.0 ± 27.2 | 30.1 ± 13.3 | 47.7 ± 12.6 | 57.8 ± 16.6 | 53.3 ± 16.6 | 44.1 ± 9.1 |
| C ₃ PC | 124 μg/kg | 7 | 120.1 ± 21.3 | 28.7 ± 14.4 | 53.4 ± 14.2 | 44.6 ± 13.1 | 43.6 ± 9.1 | 45.9 ± 8.3 |

* P < .05 vs. the control group.

** P < .01 vs. the control group.

TABLE 4

The ED₅₀ values of SOD preparations for the antagonism of a Forssman antiserum-induced increase in respiratory resistance in guinea pigs

ED₅₀ values were calculated by Probit's method.

| | ED ₅₀ (30 min after challenge) |
|----------------|---|
| | U/kg |
| Unmodified SOD | >30,000 |
| PC4-SOD | 140 |
| PC10-SOD | 240 |

We have used lipid microspheres, made of soy bean oil and PC, for drug delivery system of lipophilic drugs. Lipid microspheres have pharmacokinetic properties similar to those of liposomes (Mizushima, 1991). Liposomes and lipid microspheres both have PC as the main component of their outer layer and are highly cytotoxic. PC-SOD also demonstrated a high affinity for cells and tissues in this study, whereas a simple mixture of unmodified SOD and the PC derivative (C₃PC) produced neither a delay in plasma disappearance (unpublished data), an increase in the cellular affinity (table 1) nor an effect on Forssman antiserum-induced respiratory resistance (table 3). These results indicate that the covalent bond between C₃PC and SOD is necessary for the effective drug delivery. PC-SOD showed delayed plasma disappearance compared with unmodified SOD. This may be explained in part by the increase in molecular weight due to lecithinization, or by an increase in cell and tissue affinity.

In electrofocusing, the several spots produced by PC-SOD may have been derived from other isomers or from different PC-SOD derivatives with variable numbers of C₃PC to SOD. The determination of the positions of amide linkage between the amino group of SOD and the carboxyl group of C₃PC is under investigation.

In conclusion, the MCR values of PC-SOD were 1/3.5 to 1/6.2 times, the cell and tissue affinity of PC-SOD were 4 to 20 times and the pharmacological activities were over 200 times

more than those of unmodified SOD. It is proposed that the delayed plasma disappearance of PC-SOD and its increased affinity for tissues and cell membranes (a principal target of O₂⁻), particularly the latter, are important in the potentiation of its pharmacological activity.

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Lecithinized Superoxide Dismutase Enhances Its Pharmacologic Potency by Increasing Its Cell Membrane Affinity

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ABSTRACT

We performed the present study to clarify whether lecithinized superoxide dismutase (PC-SOD) enhanced its pharmacologic potency by increasing its cell membrane affinity. PC-SOD, in which 4 molecules of a phosphatidylcholine (PC) derivative were covalently bound to each dimer of recombinant human CuZn-SOD (rhCuZn-SOD), was shown to have a high membrane affinity using a laser confocal imaging technique. PC-SOD efficiently scavenged superoxide anion (O_2^-) produced by phorbol myristate acetate (PMA)-stimulated human neutrophils (IC_{50} 0.60 U/ml), and it exerted a dose-dependent scavenging effect (IC_{50} 1.27 U/ml) even when the neutrophils were washed after incubation with PC-SOD. In contrast, neither unmodified SOD nor polyethylene glycol-bound SOD (PEG-SOD) showed a

scavenging effect for washed neutrophils, even at a high concentration (100 U/ml). PC-SOD also showed a strong protective effect against human vascular endothelial cell damage caused by O_2^- generated by stimulated neutrophils, and PC-SOD was approximately 100-fold more potent than unmodified SOD (*in vitro* IC_{50} 100 U/ml for PC-SOD and $>10,000$ U/ml for unmodified SOD). Moreover, PC-SOD (50,000 U/kg) had an inhibitory effect on ischemia-reperfusion paw edema in mice, whereas neither unmodified SOD nor PEG-SOD had any effect. These results suggest that PC-SOD (designed to target for cell membranes) exerted a far higher pharmacologic activity by increasing cell membrane affinity than unmodified SOD and may be potentially useful for various clinical applications.

SOD is an enzyme that scavenges O_2^- , and various attempts have been made to use it in the treatment of oxygen radical-related diseases. In the United States, premature infants with incubator lung have been treated using the intratracheal instillation of rhCuZn-SOD (Davis *et al.*, 1993). However, the clinical application of SOD has been limited by its low cell membrane or tissue affinity and its rapid metabolism. Therefore, chemically modified preparations of SOD have been investigated to overcome these problems (Igarashi *et al.*, 1992; Oda *et al.*, 1989; Ogino *et al.*, 1988; Veronese *et al.*, 1983). We have previously reported on the synthesis of PC-SOD, a preparation in which rhCuZn-SOD is covalently bound to a PC derivative. Our studies have shown that PC-SOD has a higher tissue accumulation than unmodified SOD as well as a longer blood half-life and a greater inhibitory effect on the guinea pig airway response to Forssman antiserum (Igarashi *et al.*, 1992).

In the present study, the relationship between the cell

membrane affinity of PC-SOD and its pharmacologic activity was investigated using human vascular endothelial cells and neutrophils, both of which are believed to be involved in tissue injury related to oxygen radicals (McCord, 1985; Phan *et al.*, 1989; Sacks *et al.*, 1978; Zweier *et al.*, 1988), to determine whether increasing the cell membrane affinity of SOD could enhance its cytoprotective and radical scavenging activities.

Materials and Methods

Materials. Endothelial cells of human umbilical vein were purchased from Kurabo Co., Ltd. (Osaka, Japan), MCDB 107 medium from Kyokuto Co., Ltd. (Tokyo, Japan), bovine brain acidic fibroblast growth factor and fluorescein isothiocyanate-labeled anti-mouse IgG (goat) antibody from Seikagaku Kogyo Ltd. (Tokyo, Japan) and fetal calf serum from Sanko Jyunyaku Co., Ltd. (Tokyo, Japan). L-Glutamine was obtained from Boehringer Mannheim (Germany), 24-well culture plates and collagen cell disks were from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan), HBSS and PBS (pH 7.3) were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), cell culture inserts

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ABBREVIATIONS: SOD, superoxide dismutase; rhCuZn-SOD, recombinant human CuZn-SOD; PC, lecithin; phosphatidylcholine; PC-SOD, lecithinized SOD, i.e., rhCuZn-SOD covalently bound to 4 molecules of a PC palmitoyl derivative; O_2^- , superoxide anion; HBSS, Hank's balanced salt solution; PMA, phorbol myristate acetate; PEG-SOD, polyethylene glycol-bound SOD; MCDB, Molecular Cellular Developmental Biology.

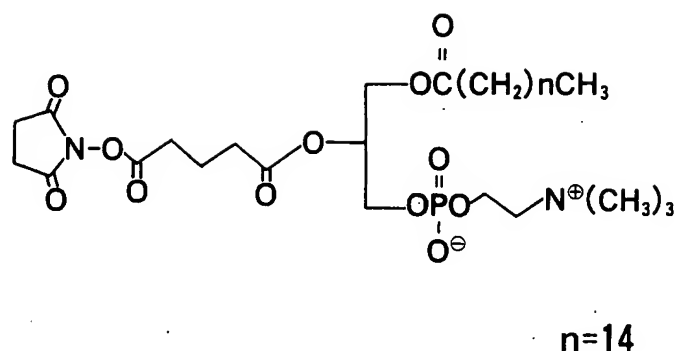


Fig. 1. Structure of activated PC palmitoyl.

(9 mm in diameter, with a pore size of 3.0 μm) were from Falcon, Becton Dickinson Labware (Lincoln Park, NJ) and lysophosphatidylcholine palmitoyl was from Sigma Chemical Co. (St. Louis, MO). Formalin, Triton X-100, PMA and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

rhCuZn-SOD and a mouse anti-CuZn-SOD monoclonal antibody (lot N6 from mouse ascites) were kind gifts from Ube Kosan Co., Ltd. (Yamaguchi, Japan), and PEG-SOD (3400 U/mg, 4 molecules of MW 3000 PEG covalently bound to each SOD dimer) was a generous gift from the Central Research Laboratories of Ajinomoto Co., Inc. (Kawasaki, Japan).

Synthesis of PC-SOD. PC-SOD was synthesized according to a method reported previously (Igarashi *et al.*, 1992). In the present study, to simplify the standardization of our PC-SOD preparation for clinical application, an activated PC palmitoyl derivative with an alkyl side chain ($n = 14$) (fig. 1) was used. *In vitro* SOD activity of PC-SOD was measured using the xanthine-xanthine oxidase method (McCord and Fridovich, 1969). The method of Lowry was used to measure the concentration of SOD (Lowry *et al.*, 1951). The number of PC molecules bound to each SOD molecule was determined by calculation of the ratio of phosphorus (P) and zinc (Zn) or copper (Cu) using an inductively coupled radiofrequency plasma emission spectrophotometer (SPS-4000, Seiko Instrument Co., Ltd., Tokyo, Japan).

Localization of PC-SOD in human endothelial cells. Collagen cell disks were inserted into 24-well culture dishes, and human

umbilical vascular endothelial cells were cultured at 37°C in a 5% CO_2 atmosphere at 2×10^4 /well in MCDB 107 medium with 20% fetal calf serum, 25 ng/ml bovine brain acidic fibroblast growth factor and 2 mM L-glutamine. At confluence (after 4 days), PC-SOD or unmodified SOD was added at a final concentration of 100 $\mu\text{g}/\text{ml}$, and the cells were incubated further for 0.5, 1 and 3 hr at 37°C. Then the cells were washed with PBS (pH 7.3), fixed with 10% neutral formalin, treated with 0.1% Triton X for 5 min and preblocked with 10% normal goat serum. Subsequently, the cells were incubated for 1 hr with a mouse anti-SOD antibody (120 $\mu\text{g}/\text{ml}$), followed by incubation for 1 hr with a fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody (15 $\mu\text{g}/\text{ml}$). As controls, the cells were incubated with a normal mouse IgG instead of the mouse anti-SOD antibody. Then, the cells were scanned with a laser confocal imaging system (MRC 600, Bio-Rad, Richmond, CA) under the following conditions: gain, 7 (0); stop, 1.5; black level, 5 (50).

Localization of PC-SOD in human neutrophils. Human neutrophils were separated from heparinized peripheral blood according to the method of Peterson *et al.* (1976) and suspended at $2 \times 10^6/\text{ml}$ in MCDB 107 medium with 10% fetal calf serum. PC-SOD or unmodified SOD was added at a final concentration of 100 $\mu\text{g}/\text{ml}$, and incubation was performed for 1 hr at 37°C in a 5% CO_2 atmosphere. Then, the cells were washed with PBS (pH 7.3) and fixed on slide glasses by centrifugation at 800 rpm for 3 min using Auto Smear CF-120 (Sakura Seiki Co., Ltd., Tokyo, Japan). The smears were subsequently treated in the manner described above for endothelial cells and then scanned with a laser confocal imaging system under the following conditions: gain, 6 (40); stop, 5; black level, 5 (50).

Effect of PC-SOD on human endothelial cell injury caused by PMA-stimulated neutrophils. Cell culture inserts were placed into 24-well culture dishes, and human umbilical vascular endothelial cells were added at 4×10^4 /well according to the method of Damle and Doyle (1989). When the cells reached confluence, 200 μl of a suspension of human neutrophils ($6 \times 10^6/\text{ml}$) in HBSS and 200 μl of PC-SOD or unmodified SOD in HBSS were added, and preincubation was performed for 1 hr at 37°C. Then, 20 μl of 22 mg/ml fluorescein isothiocyanate-labeled albumin and 20 μl of 2.2 $\mu\text{g}/\text{ml}$ PMA were added, and incubation was continued for 2 hr at 37°C. The fluorescence intensity of a 400- μl aliquot of the lower layer of the cell insert was measured using FP-777 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo) under the following conditions: excitation

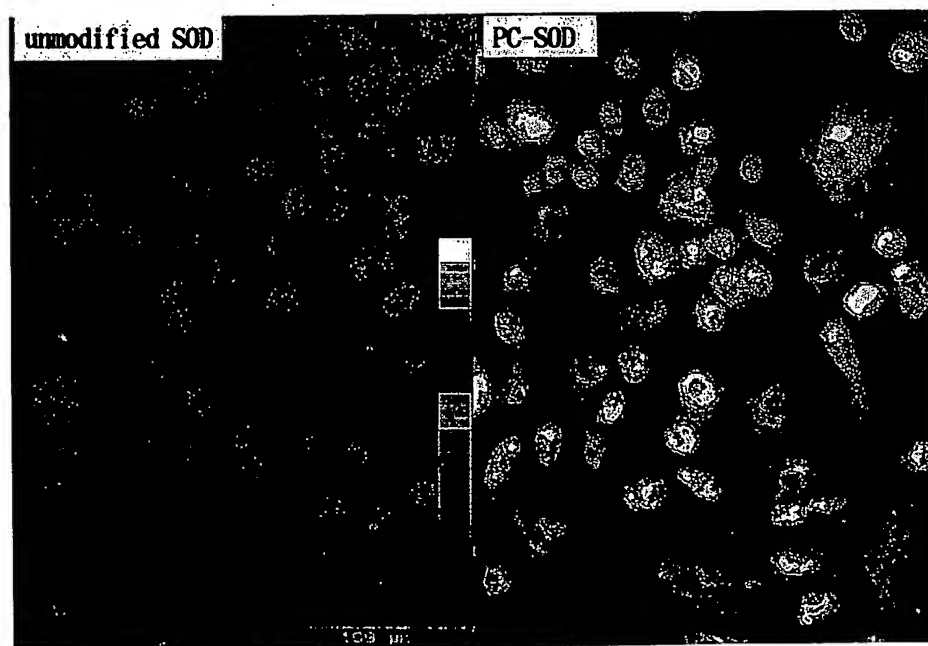


Fig. 2. Laser confocal imaging photographs of human vascular endothelial cells incubated with PC-SOD (right) or unmodified SOD (left) ($\times 20$). Human vascular endothelial cells were incubated with PC-SOD or unmodified SOD for 3 hr. Cells incubated with PC-SOD show prominent fluorescence indicating SOD uptake, whereas those incubated with unmodified SOD show trace fluorescence.

at 492 nm and emission at 520 nm to calculate the leakage of fluorescein-labeled albumin as an index of endothelial cell injury. The inhibitory effect of PC-SOD or unmodified SOD on cell damage was calculated by comparison with the leakage observed in the absence of SOD.

Scavenging effect of SOD preparation on O_2^- produced by PMA-stimulated neutrophils. Human neutrophils were suspended in HBSS at $6 \times 10^6/\text{ml}$. To 250 μl of this neutrophils suspension, 250 μl of PC-SOD, PEG-SOD or unmodified SOD in HBSS was added, and preincubation was performed for 1 hr at 37°C in a 5% CO_2 atmosphere. Then, 25 μl of 1.65 mM cytochrome C and 5 μl of 10 $\mu\text{g}/\text{ml}$ PMA were added, incubation was continued for 3 min at 37°C and the mixture was cooled on ice to terminate the reaction. Next, the mixture was centrifuged at $1,500 \times g$ for 5 min, and the optical density of the supernatant was measured at 550 nm to evaluate the scavenging effect of the SOD preparations on O_2^- produced by neu-

trophils in comparison with HBSS without SOD (Stenson *et al.*, 1984).

In other experiments, after preincubation of neutrophils with each SOD preparation, the cells were washed with HBSS and resuspended at $3 \times 10^6/\text{ml}$. Then, the procedures described above were performed to assess the scavenging effect on O_2^- .

Inhibitory effect of PC-SOD on ischemic paw edema in mice. The method of Oyanagui *et al.* (1988) was used with slight modifications. In 6-week-old male ICR mice, a rubber ring 40 mm in diameter was wound 5 times around the right hind paw at 5 min after the intravenous injection of PC-SOD, PEG-SOD or unmodified SOD (5,000, 15,000 and 50,000 U/kg as SOD, respectively). The rubber ring was removed after 20 min, reperfusion was allowed for 30 min and the paw thickness was measured with a gauge. The percentage increase over the preischemic thickness was determined as an indicator of edema.

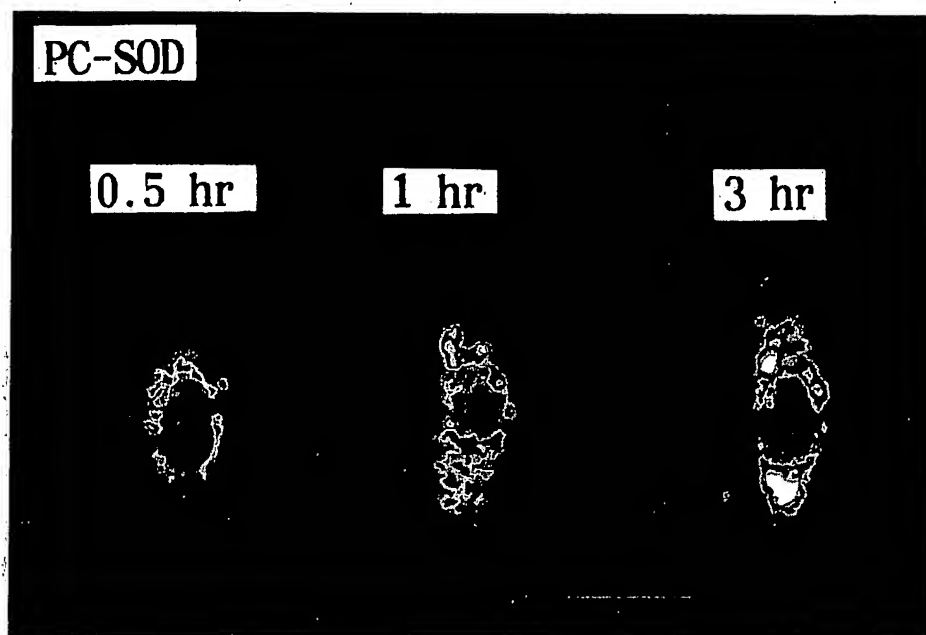


Fig. 3. Enlarged laser confocal imaging photographs of human umbilical bascular endothelial cells incubated with PC-SOD. Localization of PC-SOD was restricted to the cell membrane at 30 min, but cytoplasmic uptake was seen at 1 and 3 hr.

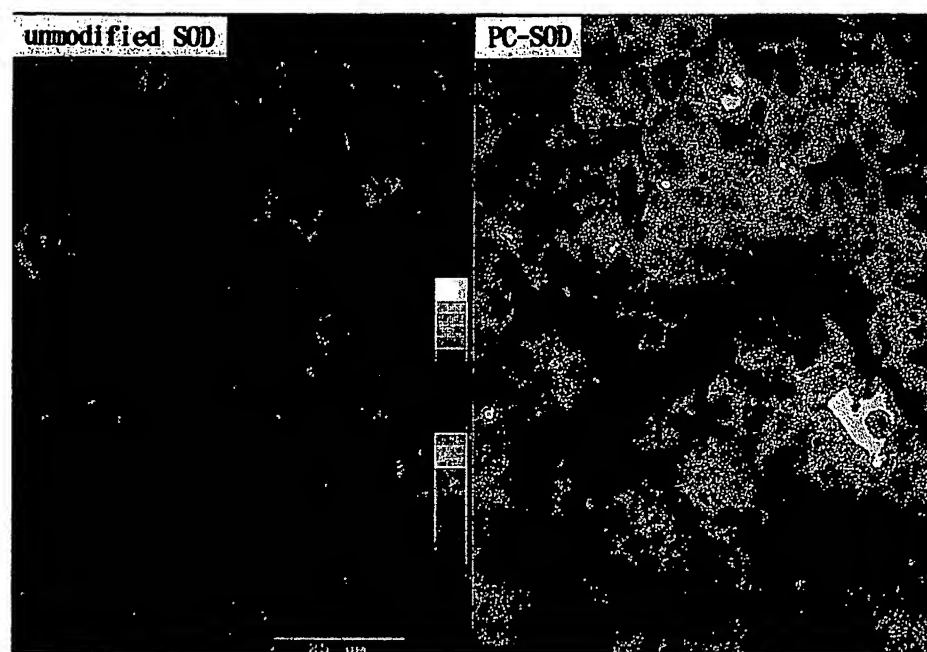


Fig. 4. Laser confocal imaging photographs of human neutrophils incubated with PC-SOD (right) or unmodified SOD (left) ($\times 60$). Cells incubated for 1 hr with PC-SOD show far greater fluorescence (indicating SOD uptake) than cells incubated with unmodified SOD.

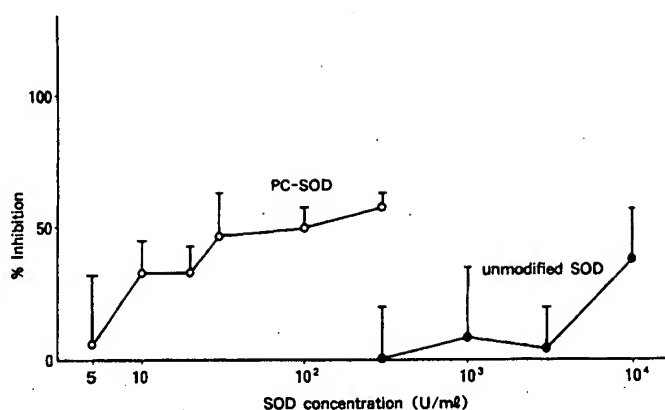


Fig. 5. Protective effect of PC-SOD against vascular endothelial cell injury caused PMA-stimulated neutrophils (mean \pm S.E.M., $n = 5$). Human umbilical vascular endothelial cells were cultured on cell culture inserts in 24-well culture dishes. At confluence, human neutrophils were added as well as PC-SOD or unmodified SOD. After incubation for 1 hr, PMA and fluorescein isothiocyanate-labeled albumin were added, and incubation was continued for another 2 hr. The fluorescence leaking out into the lower layer of the cell inserts was measured as an index of cellular injury, and the inhibitory effect of each SOD preparation was compared with that of HBSS alone to determine the percent inhibition.

Statistical analysis. Statistical analysis was performed using multiple Student's *t* test with the Bonferroni correction. The IC_{50} values were obtained using the Probit method.

The animal studies in this report were approved by the Animal Experimentation Committee of St. Marianna University.

Results

PC-SOD. The *in vitro* activity of PC-SOD by xanthine-xanthine oxidase method was 3,554 U/mg (5,180 U/mg for unmodified SOD), and an average of 4 molecules of a PC derivative were bound to each SOD dimer.

Localization of PC-SOD in endothelial cells. Laser confocal imaging revealed the marked uptake of SOD by endothelial cells incubated with PC-SOD (fig. 2). SOD accumulated in the cell membranes after 0.5 hr and in the cytoplasm after 1 and 3 hr (fig. 3). On the other hand, unmodified SOD showed trace accumulation in the endothelial cells even after 3 hr of incubation (fig. 2). The control cells incubated with normal mouse IgG instead of anti-SOD antibody did not show any fluorescence.

TABLE 1

Scavenging effect of SOD preparations on O_2^- produced by PMA-stimulated human neutrophils

The percent scavenging effect of each SOD preparation was calculated in comparison with O_2^- production when HBSS was added instead of SOD preparation.

| | 0.1 U/ml | 1.0 U/ml | 10 U/ml | IC_{50} |
|----------------|----------------|-----------------|-------------------|-----------|
| | | % | | U/ml |
| PC-SOD | 10.8 \pm 7.4 | 55.1 \pm 2.0§ | 100.2 \pm 1.7†§ | 0.60 |
| PEG-SOD | — | 13.2 \pm 4.1 | 73.6 \pm 1.7 | 4.32 |
| Unmodified SOD | 1.4 \pm 8.0 | 24.8 \pm 8.0 | 80.8 \pm 2.6 | 2.73 |

Data show the percent scavenging effect *without washing* neutrophils after preincubation as the mean \pm S.E.M. of 5 experiments.

| | 0.1 U/ml | 1.0 U/ml | 10 U/ml | 100 U/ml | IC_{50} |
|----------------|----------------|------------------|------------------|------------------|-----------|
| | | % | | | U/ml |
| PC-SOD | 14.7 \pm 7.6 | 44.2 \pm 8.6†‡ | 85.0 \pm 7.7†§ | 94.8 \pm 4.2†§ | 1.27 |
| PEG-SOD | — | 8.1 \pm 6.5 | 5.0 \pm 4.5 | 9.2 \pm 5.0 | >100 |
| Unmodified SOD | — | 12.3 \pm 3.3 | 11.5 \pm 3.4 | 9.3 \pm 6.5 | >100 |

Data show the percent scavenging effect *with washing* neutrophils after preparation as the mean \pm S.E.M. of 4 experiments.

* $P < .05$, † $P < .001$ vs. unmodified SOD.

‡ $P < .05$, § $P < .001$ vs. PEG-SOD.

Localization of PC-SOD in neutrophils. Figure 4 shows laser confocal images obtained after the incubation of neutrophils with PC-SOD or unmodified SOD for 1 hr. PC-SOD showed a similar uptake by neutrophils as by endothelial cells. PC-SOD was mainly localized in the cell membranes, although this was not as clear as in the endothelial cells because of distortion of the neutrophils by fixation and smearing. In contrast, unmodified SOD showed trace uptake as with endothelial cells. The cells treated with a normal mouse IgG did not show any fluorescence.

Protective effect of PC-SOD on vascular endothelial cell injury. PC-SOD showed a far more potent protective effect against vascular endothelial cell injury caused by PMA-stimulated neutrophils compared with unmodified SOD (fig. 5). The IC_{50} was approximately 100 U/ml for PC-SOD and more than 10,000 U/ml for unmodified SOD.

Scavenging effect of the SOD preparations. When PMA was added to neutrophil suspensions without washing the cells after preincubation with the SOD preparations, PC-SOD showed a higher scavenging effect on O_2^- than unmodified SOD and PEG-SOD (IC_{50} 0.60 U/ml for PC-SOD, 2.73 U/ml for unmodified SOD and 4.32 U/ml for PEG-SOD), and its scavenging effect was significantly greater at a concentration of 1.0 U/ml and 10 U/ml ($P < .001$) (table 1). When PMA was added to neutrophils that were washed after the preincubation with each SOD preparation, PC-SOD showed a dose-dependent scavenging activity (IC_{50} 1.27 U/ml). For the washed neutrophil suspension, neither unmodified SOD nor PEG-SOD had a scavenging effect at the concentration tested.

Inhibitory effect of PC-SOD on ischemic paw edema. PC-SOD had a significant inhibitory effect on ischemia-reperfusion paw edema in mice at 50,000 U/kg ($P < .001$ vs. the control group), whereas neither unmodified SOD nor PEG-SOD had an inhibitory effect (fig. 6).

Discussion

Active oxygen species such as O_2^- have been reported to be involved in tissue injury related to ischemia-reperfusion myocardial infarction (Gardner, 1983), Crohn's disease (Sue-matsu, 1987), allergic diseases (Brigham, 1986) and oncogenesis. Hereditary insufficiency of SOD activity has also been

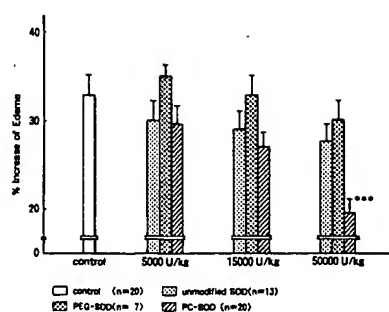


Fig. 6. Inhibitory effect of PC-SOD on mouse paw edema produced by ischemia-reperfusion injury (mean \pm S.E.M.). Five minutes after the intravenous injection of each SOD preparation, a rubber ring was wound around the right hind paw. After 20 min of ischemia, the ring was removed, and 30 min later the paw thickness was measured with a gauge. Severity of edema was determined on the basis of the percent increase over the preischemic paw thickness. *** $P < .001$ vs. control.

suggested to be involved in amyotrophic lateral sclerosis (Rosen *et al.*, 1993). Not only does O_2^- have a direct cytotoxic effect, but it also is the precursor of hydrogen peroxide and the potentially cytotoxic hydroxyl radical. Recently, it has been reported that O_2^- shortens the life of endothelium-dependent relaxing factor (believed to be nitric oxide) (Grylewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986) and that hydroxyl radicals might be produced from O_2^- and nitric oxide (Beckman *et al.*, 1990). Thus, the elimination of O_2^- may be important for the control of oxygen radical-related diseases.

CuZn-SOD and Mn-SOD are normally present in cells at a sufficient level to scavenge intracellular O_2^- (Marklund, 1984). Although extracellular SOD was reported as a protector in the extracellular space and endothelial cell surface, the activity of extracellular SOD is low (Karlsson and Marklund, 1989; Marklund, 1984). These suggest that exogenous SOD may be of clinical value by scavenging excess O_2^- at the cell membrane. Accordingly, we have focused on the development of a method of delivering SOD effectively to the cell membrane, and we produced PC-SOD for this purpose (Igarashi *et al.*, 1992). The present study clearly indicated that PC-SOD had a high cell membrane affinity and a high cellular uptake for both endothelial cells and neutrophils. In addition, the scavenging effect of PC-SOD on O_2^- produced by PMA-stimulated neutrophils was much more potent than that of unmodified SOD or PEG-SOD; the latter is a long-acting form of SOD and currently undergoing clinical trials for use in traumatic cerebral edema (Muizelaar *et al.*, 1993). The IC_{50} of PC-SOD was 0.60 U/ml for unwashed neutrophils and 1.27 U/ml when neutrophils were washed after incubation with the SOD preparation (table 1), indicating that 50% of the activity of PC-SOD was retained by the cells after washing. In contrast, neither PEG-SOD nor unmodified SOD showed any scavenging effect on O_2^- produced by washed neutrophils. Thus, the persistent scavenging effect of PC-SOD after the washing of neutrophils probably reflected its high membrane affinity and cellular uptake.

PC-SOD was 100 times more potent than unmodified SOD (IC_{50} 100 U/ml for PC-SOD and more than 10,000 U/ml for unmodified SOD) in blocking endothelial cell damage by PMA-stimulated neutrophils. In addition, PC-SOD was more effective than unmodified SOD and PEG-SOD in the suppression of ischemic mouse paw edema, a phenomenon in which neutrophils and endothelial cells are considerably involved

(Oyanagui *et al.*, 1988). Moreover, PC-SOD showed a more potent scavenging effect on O_2^- produced by neutrophils and a stronger inhibitory effect on mouse paw edema than other types of chemically modified SOD, such as stylenecomaleic acid butylester-bound SOD (Ogino *et al.*, 1988) and pyranco-polymer-bound SOD (Oda *et al.*, 1989), although data were not given in this article. We compare the activity of PC-SOD with that of extracellular SOD or SOD having heparin-binding peptide (Inoue *et al.*, 1990).

Toxicological and antigenic studies of PC-SOD showed no particular findings in comparison with unmodified SOD (data not shown). Accordingly, PC-SOD has the potential to be used in the treatment of various diseases.

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Lecithinized copper,zinc-superoxide dismutase as a protector against doxorubicin-induced cardiotoxicity in mice

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Abstract

Production of superoxide radicals from doxorubicin is widely accepted to be the cause of the cardiotoxicity induced by this antitumor agent. Pretreatment with superoxide dismutase could improve the therapeutic application. Aim of the present study was to determine whether lecithinized superoxide dismutase (PC-SOD) can serve as a cardioprotective drug during doxorubicin treatment. The protective potential of PC-SOD on doxorubicin-induced cardiotoxicity was investigated in BALB/c mice. The possible influence of PC-SOD on the antitumor activity of doxorubicin was investigated in vitro as well as in vivo.

Mice were treated intravenously with doxorubicin (4 mg·kg⁻¹) or doxorubicin and PC-SOD (5000, 20000 or 80000 U·kg⁻¹) weekly × 6 and appropriate controls were included. Cardiotoxicity was monitored for 8 weeks by ECG measurement. The influence of PC-SOD on the antitumor activity of doxorubicin was evaluated in three human malignant cell lines. Nude mice bearing OVCAR-3 human ovarian cancer xenografts were treated intravenously with doxorubicin (8 mg·kg⁻¹) alone or preceded by PC-SOD 20000 or 80000 U·kg⁻¹ weekly × 2 and appropriate controls were included. PC-SOD prevented doxorubicin-induced cardiotoxicity already at 5000 U·kg⁻¹ whereas 20000 and 80000 U·kg⁻¹ were equally protective. No toxicity was observed in mice treated with PC-SOD. PC-SOD did not interfere with the antiproliferative effects of doxorubicin in vitro. In vivo, PC-SOD had no negative effect on the inhibition of xenograft growth induced by doxorubicin.

It can be concluded that PC-SOD protects the heart, but not the tumor against doxorubicin. These data suggest that PC-SOD may be a suitable cardioprotector during doxorubicin treatment.

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Keywords: Doxorubicin; Antitumor effect; Cardiotoxicity; Superoxide radical; Lecithinized superoxide dismutase, PC-SOD; Mouse; Electrocardiogram

Introduction

The involvement of oxygen radicals such as superoxide anions (O₂⁻) in doxorubicin-induced cardiotoxicity is widely accepted (Keizer et al., 1990; Olson and Mushlin, 1990; Powis, 1987). This cardiotoxicity, which manifests itself as congestive heart failure even years after completion of treatment, limits the maximum cumulative dose of doxorubicin that can be administered. Redox cycling of

doxorubicin (Fig. 1) generates oxygen radicals. Doxorubicin is enzymatically reduced to the doxorubicin semiquinone radical by NADPH and cytochrome P450 reductase, NADH and NADPH dehydrogenase, or cytochrome b₅ reductase (Julicher et al., 1986; Powis, 1987; Sterenberg et al., 1986). This semiquinone radical directly donates its extra electron to molecular oxygen, yielding O₂⁻. Copper,zinc-superoxide dismutase (SOD1) is normally present in sufficient amounts to keep intracellular concentrations of O₂⁻ low. However, excessive O₂⁻ production may overwhelm the defense system leading to doxorubicin-induced cardiotoxicity (Sarvazyan et al., 1995). Supplementing with exogenous SOD1 appears to be a rational remedy against increased production of O₂⁻, but the clinical application of SOD1 has thus far been limited by its low cell membrane affinity and its rapid

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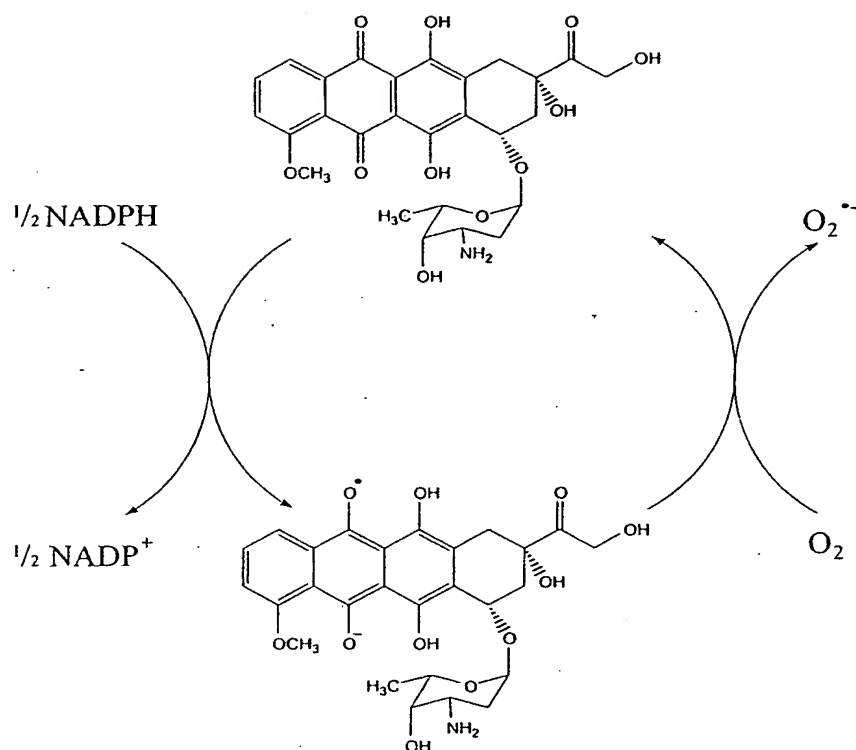


Fig. 1. Doxorubicin redox cycling. Doxorubicin is enzymatically reduced to the doxorubicin semiquinone radical. This semiquinone radical is subsequently oxidized by oxygen yielding doxorubicin and superoxide anion.

clearance (Fujita et al., 1992). In order to increase the cell membrane affinity and the plasma half-life of SOD1, Igarashi et al. (1992, 1994) have developed lecithinized SOD1 (PC-SOD), in which four molecules of a phosphatidylcholine (PC) derivative are covalently bound to recombinant human SOD1. It has been reported that PC-SOD at dose levels of $50\,000 \text{ U}\cdot\text{kg}^{-1}$ was effective in several animal models for inflammatory diseases, such as the dextran sulfate sodium-induced rat colitis model (Hori et al., 1997) and in the bleomycin-induced interstitial pneumonia mouse model (Tamagawa et al., 2000; Yamazaki et al., 1997). Because of the alleged role of $\text{O}_2^{\bullet -}$ in doxorubicin-induced toxicity, it is expected that PC-SOD protects the heart during doxorubicin treatment. The aim of the present investigation was to evaluate the potential of PC-SOD as a cardioprotective compound during doxorubicin treatment in the mouse. Furthermore, interference of PC-SOD with the antitumor activity of doxorubicin was investigated both *in vitro* and *in vivo*.

Methods

Chemicals. PC-SOD was provided by Seikagaku Corporation (Tokyo, Japan). Doxorubicin (Adriablastina) was obtained from Pharmacia Nederland BV (Woerden, The Netherlands). PC-SOD was dissolved in 5% mannitol solution. Doxorubicin was dissolved to a concentration of 2

$\text{mg}\cdot\text{ml}^{-1}$ in saline. Doxorubicin for the *in vitro* experiments was further diluted in complete cell culture medium.

Effect of PC-SOD on doxorubicin-induced cardiotoxicity.

The protocol was approved by the ethics committee for animal experiments of the Maastricht University. In male BALB/c mice, ECG sensing telemeters (DataScience International, St. Paul, USA) were implanted subcutaneously. After a recovery period of 2 weeks, the mice were submitted to one of the following weekly dose schedules for 6 weeks:

- Group 1 ($n = 3$): 0.9% NaCl solution *i.v.*
- Group 2 ($n = 6$): doxorubicin $4 \text{ mg}\cdot\text{kg}^{-1}$ *i.v.*
- Group 3 ($n = 6$): PC-SOD $5000 \text{ U}\cdot\text{kg}^{-1}$ *i.v.*, followed by doxorubicin $4 \text{ mg}\cdot\text{kg}^{-1}$ after 15 min
- Group 4 ($n = 6$): PC-SOD $20\,000 \text{ U}\cdot\text{kg}^{-1}$ *i.v.*, followed by doxorubicin $4 \text{ mg}\cdot\text{kg}^{-1}$ after 15 min
- Group 5 ($n = 6$): PC-SOD $80\,000 \text{ U}\cdot\text{kg}^{-1}$ *i.v.*, followed by doxorubicin $4 \text{ mg}\cdot\text{kg}^{-1}$ after 15 min
- Group 6 ($n = 6$): 5% mannitol solution *i.v.*, followed by doxorubicin $4 \text{ mg}\cdot\text{kg}^{-1}$ after 15 min.

The doses of PC-SOD used in this experiment were based on the observation of Yamazaki et al. (1997) who observed that $50\,000 \text{ U}\cdot\text{kg}^{-1}$ was necessary to suppress the progression of pulmonary fibrosis in the bleomycin-induced interstitial pneumonia mouse model. All injections were administered on the first day of each week via

the tail vein. After 6 weeks of treatment, animals were observed for another 2 weeks. The ECG was registered in the freely moving animal on the second day after treatment and on the same day of the week during the observation period. For interpretation of the ECG, nine complexes were analyzed in detail. The ST interval was defined as the period from the S-peak to the end of the T-peak in milliseconds (Fig. 2). Increases in ST interval length were expressed as mean \pm SEM. The data were evaluated by a two-way ANOVA with time and treatment as independent variables. Post hoc multiple comparisons for time and treatment group were performed with Fisher's LSD test. The software used was SPSS for Windows 10.1 (SPSS Inc., Chicago, USA).

After the observation period, mice were terminated, the hearts were excised and taken into buffered formalin

for 24 h and stored in 70% ethanol until processing. Hearts were spliced and embedded in paraffin. Sections (4 mm) were cut and stained with hematoxylin and eosin. Myocardial damage was evaluated by light microscopy according to Billingham (1983). Specific attention was paid to the occurrence of lesions in cardiac tissue.

Effect of PC-SOD on the antitumor effect of doxorubicin.

For the in vitro part of the study, three cell lines were used: the human ovarian cancer cell lines A2780 (Eva et al., 1982) and OVCAR-3 (Hamilton et al., 1983) and the human breast cancer cell line MCF-7 (Soule et al., 1973). The cells were routinely grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, Scotland) supplemented with 10% heat inactivated fetal calf serum (GIBCO, New York, USA). Exponentially

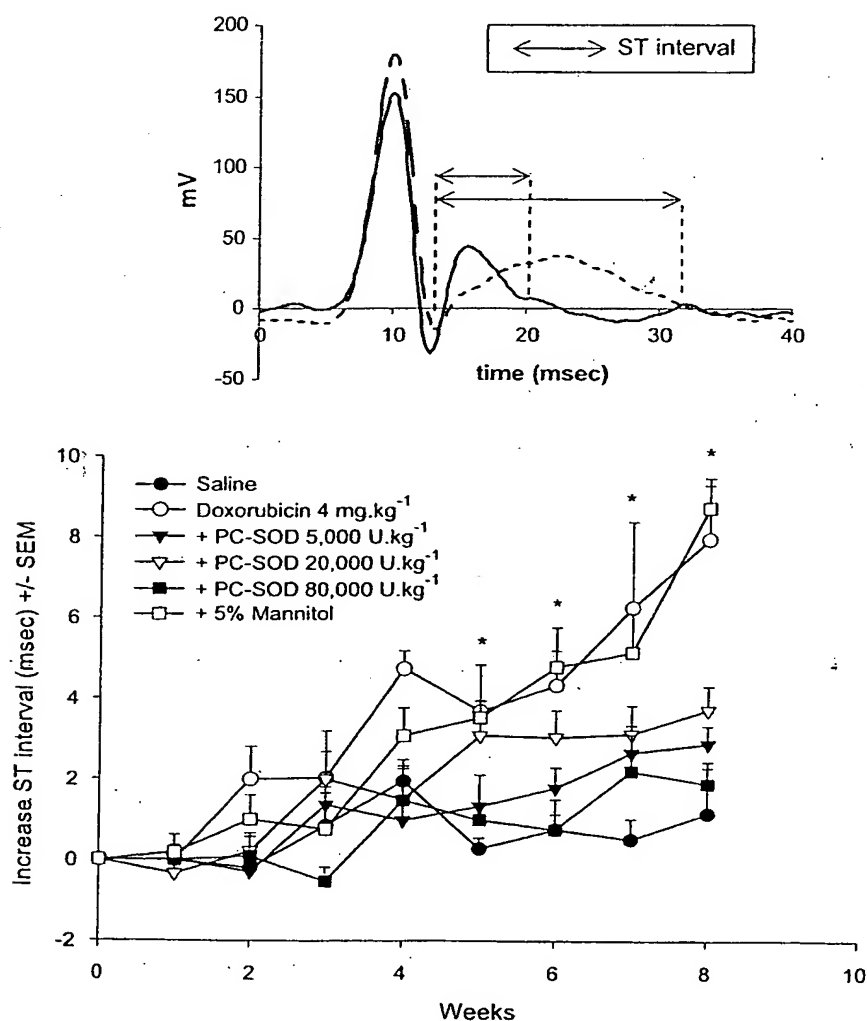


Fig. 2. Upper panel: effect of doxorubicin treatment (4 mg.kg⁻¹ weekly for 6 weeks) on the mouse ECG. The ECG recorded before treatment with doxorubicin is represented by the solid line. Treatment with doxorubicin causes flattening of the T top and an increased ST interval length in the ECG that is represented by the dashed line. Lower panel: increase in ST interval per treatment group. Mean \pm SEM ($n = 6$). * $P < 0.05$ relative to animals treated with saline.

growing cells were harvested and plated as single cell suspensions in 96-wells flat bottom microtiter plates (Greiner, Solingen, Germany). Cells were seeded in a 100- μ l medium at a density of 3000 cells per well (A2780) or 5000 cells per well (OVCAR-3 and MCF-7). After 24 h, doxorubicin was added resulting in a range of concentrations of 10 fM to 10 μ M. PC-SOD was added resulting in concentrations of 100, 400, and 1600 U·ml⁻¹. All concentrations were studied in quadruplicate wells. After 96 h, the IC₅₀ (defined as the doxorubicin concentration that decreases cell growth by 50% when compared to control cell growth) was calculated. The effect of PC-SOD on cell growth was expressed as a percentage of control cell growth. The experiments were carried out four times.

The protocol for the in vivo part of the study was approved by the ethics committee for animal experiments of the Vrije Universiteit Amsterdam. The effect of PC-SOD on the antitumor activity of doxorubicin was investigated in mice according to Boven et al. (1990). Nude female mice (Hsd:Athymic-nu) were obtained from Harlan Olac (Horst, The Netherlands) at the age of 6 weeks. The animals were maintained in isolation under controlled atmospheric conditions (temperature, 23–25 °C; humidity, 50–60%). Animal handling was carried out under sterile conditions. First, it was assessed whether PC-SOD had an influence on the weight loss in non-tumor-bearing nude mice induced by doxorubicin in groups of five mice each. Thereafter, OVCAR-3 xenograft fragments of 3-mm diameter were implanted subcutaneously into both flanks of 8-week-old nude mice. As xenografts, OVCAR-3 shows a pattern of a poorly differentiated serous adenocarcinoma and has a doubling time of approximately 5 days. Treatment was started when the tumor size was approximately 100 mm³; the first treatment day was designated as day 0. The mice were subjected to the following weekly dose schedules for 2 weeks:

- Group 1 ($n = 6$): control
- Group 2 ($n = 6$): doxorubicin 8 mg·kg⁻¹ i.v.
- Group 3 ($n = 6$): PC-SOD 80000 U·kg⁻¹ i.v.
- Group 4 ($n = 6$): PC-SOD 20000 U·kg⁻¹ i.v., followed by doxorubicin 8 mg·kg⁻¹ i.v. after 15 min
- Group 5 ($n = 6$): PC-SOD 80000 U·kg⁻¹ i.v., followed by doxorubicin 8 mg·kg⁻¹ i.v. after 15 min.

The dose of doxorubicin 8 mg·kg⁻¹ weekly \times 2 is maximally tolerated in the OVCAR-3 nude mouse xenograft model (Boven et al., 1990). Mice were weighed twice a week and weight loss was used as a measure of toxicity. Toxic deaths were defined as animals that died within 14 days after the last injection.

Tumor volume was measured in three dimensions twice a week. The antitumor activity was expressed as the percentage of growth inhibition, which is 100%–T/C%. T/C% was calculated by the ratio of the mean volume of treated

tumors/mean volume of control tumors \times 100%. Data was evaluated using a three-way ANOVA with doxorubicin dose, PC-SOD dose, and time as independent variables. Post hoc test using Fisher's LSD was performed for the variable time only.

Results

Effect of PC-SOD on doxorubicin-induced cardiotoxicity

In Fig. 2, the effect of doxorubicin alone or in combination with PC-SOD (5000, 20000 or 80000 U·kg⁻¹) on the length of the ST interval in the mouse ECG is presented. A two-way between groups ANOVA was used to examine the main effects and interactions of time and treatment on the increase in length of the ST interval. There was a significant two-way interaction between time and treatment [$F(40,216) = 2.147$, $P < 0.01$]. Significant one-way interactions between time and increase in ST interval [$F(8,216) = 23.257$, $P < 0.01$] and treatment and increase in ST interval [$F(5,216) = 22.442$, $P < 0.01$] were observed. Post hoc comparison of the means with Fisher's LSD test revealed that treatment with doxorubicin (4 mg·kg⁻¹ i.v.) for 6 weeks increased the length of the ST interval by 7.9 ± 1.3 ms at week 8 ($P < 0.01$). Treatment with saline did not cause any changes in the length of the ST interval. Treatment with mannitol, the solvent for PC-SOD, before administration of doxorubicin, did not prevent the doxorubicin-induced lengthening of the ST interval ($P = 0.201$ compared to doxorubicin only and $P < 0.01$ compared to saline). Pretreatment with 5000 U·kg⁻¹ PC-SOD completely prevented the lengthening of the ST interval ($P < 0.01$ compared to pretreatment with mannitol and $P = 0.149$ compared to treatment with saline only). Increasing the dose of PC-SOD to 20000 or 80000 U·kg⁻¹ also prevented the ST interval increase induced by doxorubicin ($P < 0.01$ compared to treatment with mannitol). The ST interval in the PC-SOD 20000 U·kg⁻¹ treatment group was, however, also longer than that in the saline-treated group ($P < 0.01$) (Fig. 2).

The hearts were removed from the mice 2 weeks after completion of treatment. Microscopic evaluation of the hematoxylin- and eosin-stained sections revealed that treatment with doxorubicin induced small lesions in cardiac tissue (Fig. 3B). In mice that received saline only, these lesions were not observed (Fig. 3A). The lesions were not observed in mice that were pretreated with PC-SOD before doxorubicin was administered (Fig. 3C).

Effect of PC-SOD on doxorubicin-induced cell growth inhibition

PC-SOD alone seemed to stimulate the proliferation in MCF-7 cells but did not affect the growth of A2780 and

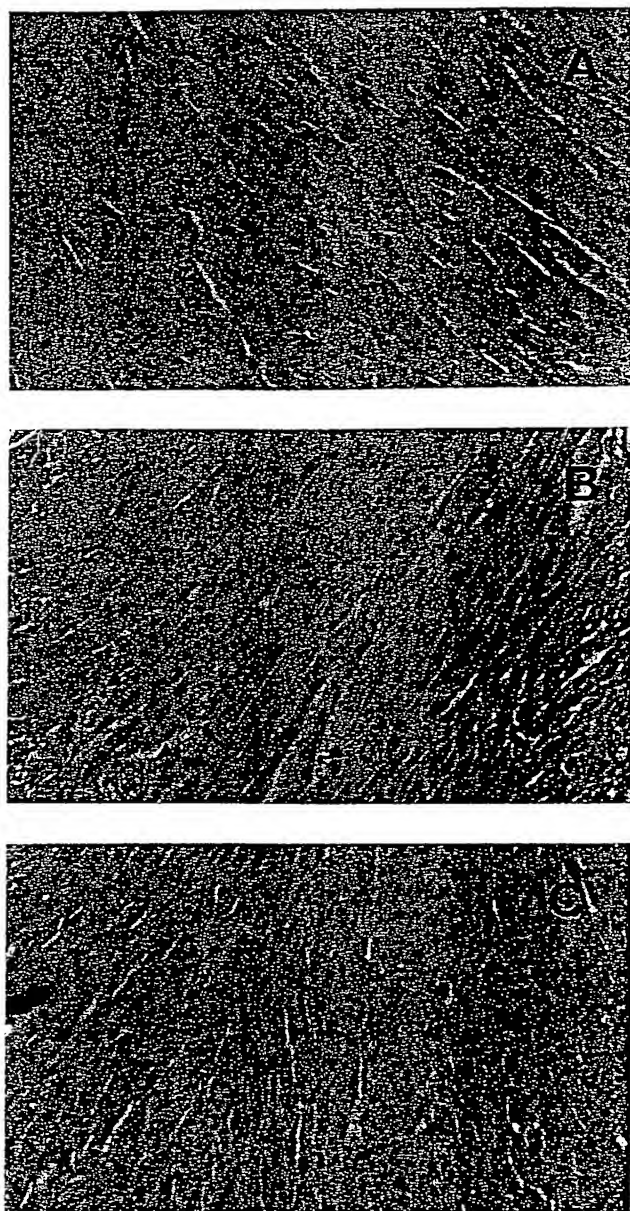


Fig. 3. Light micrographs showing hematoxylin and eosin stained 4- μ m sections of male BALB/c mouse hearts. (A) Control. (B) After treatment with doxorubicin ($4 \text{ mg} \cdot \text{kg}^{-1}$ weekly for 6 weeks). (C) After treatment with doxorubicin ($4 \text{ mg} \cdot \text{kg}^{-1}$) and PC-SOD ($80000 \text{ U} \cdot \text{kg}^{-1}$) weekly for 6 weeks. Arrows indicate the lesions in cardiac tissue.

OVCAR-3 cells (Fig. 4A). MCF-7 cells were approximately 4 times more sensitive to doxorubicin than A2780 and OVCAR-3 cells; IC_{50} 's were 4.2 ± 0.5 , 16.9 ± 4.6 , $12.6 \pm 9.3 \text{ nM}$, respectively. In A2780 cells, the inhibitory effect of doxorubicin on cell proliferation was enhanced by PC-SOD (Fig. 4B). The IC_{50} of doxorubicin on A2780 cell proliferation was decreased from $16.9 \pm 4.6 \text{ nM}$ (no PC-SOD present) to $4.7 \pm 1.9 \text{ nM}$ ($1600 \text{ U} \cdot \text{ml}^{-1}$ PC-SOD) ($P = 0.048$). In MCF-7 cells, the IC_{50}

of doxorubicin on cell proliferation was significantly increased in the presence of 30 and $120 \mu\text{g} \cdot \text{ml}^{-1}$ PC-SOD [from 4.3 ± 0.3 to $7.2 \pm 0.6 \text{ nM}$ ($P = 0.003$) and $7.9 \pm 0.7 \text{ nM}$ ($P = 0.002$), respectively]. However, the IC_{50} of doxorubicin was unchanged with PC-SOD present at the highest concentration tested ($1600 \text{ U} \cdot \text{ml}^{-1}$) (Fig. 4B). In OVCAR-3 cells, PC-SOD at all tested concentrations had no effect on the doxorubicin-induced inhibition of cell proliferation (Fig. 4B).

Effect of PC-SOD on doxorubicin-induced inhibition of tumor growth

Non-tumor-bearing nude mice treated with doxorubicin alone suffered a maximum weight loss of $5.7 \pm 3.7\%$ (Fig. 5). In nude mice that were treated with PC-SOD alone or the

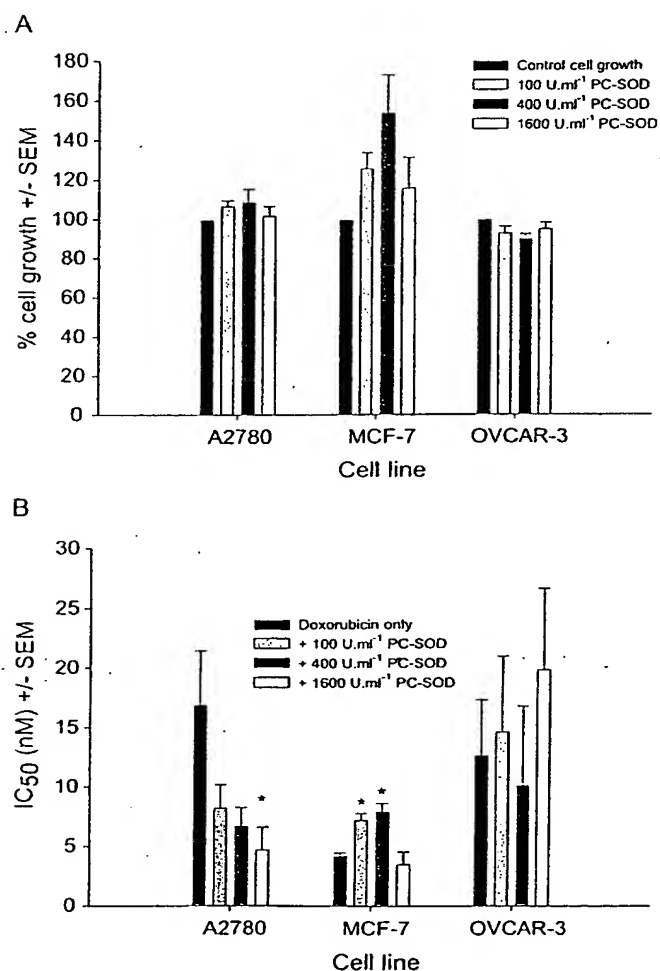


Fig. 4. (A) The effect of PC-SOD on cell proliferation in three different human malignant cell lines. Cells were exposed to PC-SOD for a period of 96 h. Control cell growth is set at 100%. Mean \pm SEM ($n = 4$). (B) The effect of PC-SOD on the IC_{50} of doxorubicin on cell proliferation in A2780, MCF-7, and OVCAR-3 tumor cell lines. Cells were exposed to doxorubicin or the combination of doxorubicin and PC-SOD for a period of 96 h. Mean \pm SEM ($n = 4$). * $P < 0.05$.

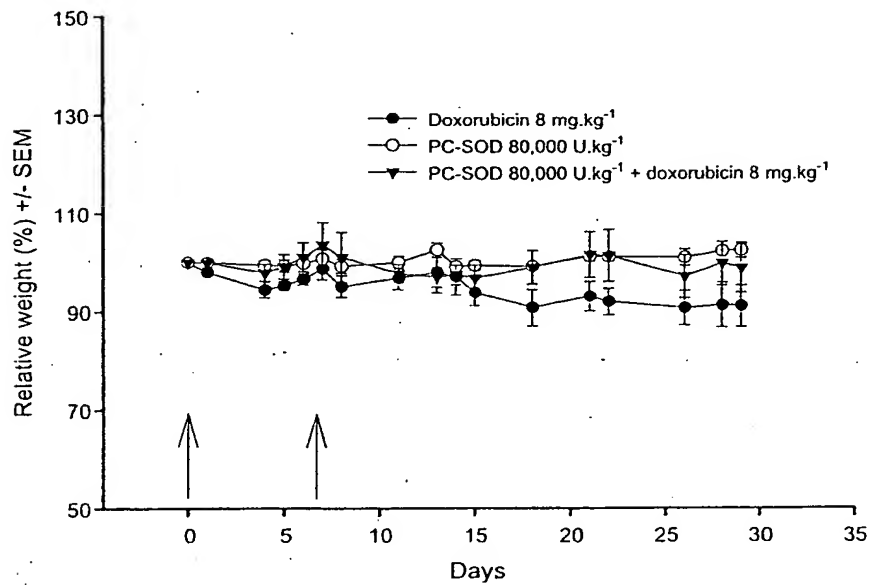


Fig. 5. The effect of doxorubicin, PC-SOD, or the combination on body weight of mice ($n = 5$). Arrows indicate treatment (day 0 and day 7). Mean \pm SEM ($n = 6$).

combination of doxorubicin and PC-SOD, maximum weight loss was $0.9 \pm 3.0\%$ and $2.2 \pm 4.1\%$, respectively (Fig. 5). In all groups, one mouse developed ascites that resulted in the death of one animal each in two groups.

Subsequently, treatment was given to nude mice bearing well established OVCAR-3 xenografts. A three-way ANOVA between groups ANOVA was used to examine the main effects and interactions of doxorubicin, PC-

SOD, and time on tumor volumes. ANOVA revealed that there was no three-way interaction among doxorubicin, PC-SOD, and time on tumor volume [$F(9,472) = 1.385$, $P = 0.192$]. There was a two-way interaction between time and treatment with doxorubicin on tumor volume [$F(14,472) = 38.549$, $P < 0.01$]. There were no two-way interactions between PC-SOD and time or dox and tumor volume. Examination of the means with Fisher's LSD

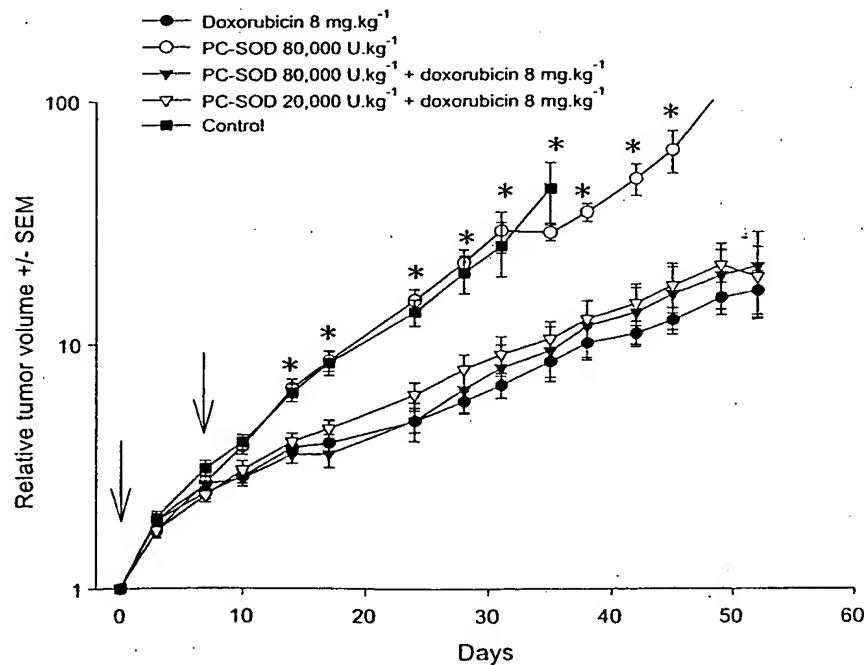


Fig. 6. The effect of doxorubicin, PC-SOD, and combination of both on relative tumor volume in mice bearing well-established OVCAR-3 xenografts. Arrows indicate treatment (day 0 and day 7). Mean \pm SEM ($n = 6$). * $P < 0.05$ relative to animals treated with doxorubicin.

test revealed that tumor volumes increased in all five groups but did so more slowly in the three groups treated with doxorubicin than in the groups that received saline only or PC-SOD only, at least after day 10 (day 14: $P = 0.011$) (Fig. 6). Tumor volumes in the group of mice that received PC-SOD alone were not different from those in mice that received saline (Fig. 6). In the group of PC-SOD 20000 U·kg⁻¹ + doxorubicin, one mouse died from ascites.

Discussion

Cardiotoxicity caused by treatment with doxorubicin can be life-threatening and may occur even years after completion of therapy (Kapusta et al., 2000). It is widely accepted that oxygen-free radicals generated during doxorubicin redox cycling are responsible for the damage that doxorubicin causes to the heart (Julicher et al., 1985; Singal and Iliskovic, 1998; van Acker et al., 1997; Venditti et al., 1998). Oxygen radical generation affects the heart because doxorubicin and its toxic metabolite doxorubicinol accumulate in cardiac tissue that has low antioxidant levels (de Jong et al., 1991). Pretreatment with antioxidants is therefore a rational approach to prevent doxorubicin-induced cardiotoxicity. Antioxidant cardioprotectors such as the flavonoid monoHER (van Acker et al., 1995), the lipid lowering agent probucol (Singal et al., 1995), and the iron chelator ICRF-187 (Koning et al., 1991) have been shown to be successful in several animal models (Herman and Ferrans, 1998; van Acker et al., 1995).

We have investigated the cardioprotective efficacy of PC-SOD using the telemetry model in mice as described earlier by van Acker et al. (1996a, 1996b). In this model, the toxic effect of doxorubicin on the heart is monitored by measuring the length of the ST interval in the mouse ECG using implanted telemeters. A good correlation has been demonstrated between the extent of tissue damage and ST interval prolongation as a result of the total cumulative doxorubicin dosage administered (van Acker et al., 1996b; Villani et al., 1986). A compound that attenuates doxorubicin-induced damage to cardiac tissue is therefore expected to inhibit doxorubicin-induced ST interval prolongation. By ECG measurements, we have demonstrated that PC-SOD reduces cardiac damage in mice treated with doxorubicin. This protective activity of PC-SOD was confirmed by histological evaluation of cardiac tissue from mice that received saline, doxorubicin, or doxorubicin combined with PC-SOD. To our knowledge, PC-SOD is the first semisynthetic enzymatic antioxidant that has been shown to protect against doxorubicin-induced cardiotoxicity.

Furthermore, we have demonstrated that PC-SOD is already effective at dose of 5000 U·kg⁻¹. Pretreatment with higher doses of PC-SOD (20000 and 80000 U·kg⁻¹) did not increase cardioprotection. Apparently, PC-SOD is that potent in our model that a dose-dependency for the cardi-

oprotection offered by PC-SOD could not be shown. PC-SOD was well tolerated and did not cause any signs of general toxicity or loss of body weight. Therefore, PC-SOD appears to be suitable as a cardioprotector during treatment with doxorubicin.

Several SOD-related strategies (administration of SOD1, SOD2 or SOD mimetics, or targeted gene delivery of SOD via viral vectors) have been proposed for the prevention of doxorubicin-induced cardiotoxicity. The main strength of the approach with PC-SOD is that this compound remains in the circulation for a longer time (Igarashi et al., 1992, 1994). Furthermore, Hangaishi et al. (2001) have demonstrated that treatment of rats with PC-SOD results in an elevated SOD activity in cardiac tissue. In addition to doxorubicin-induced cardiotoxicity, superoxide anions also play a prominent role in ischemia-reperfusion-induced injury. Treatment with PC-SOD limited the infarct size following ischemia-reperfusion (Hangaishi et al., 2001).

A main prerequisite for a compound to be used as a cardioprotector during treatment for cancer is that it does not interfere with the antitumor activity of the chemotherapy (van Acker et al., 1997). In order to evaluate the possible clinical application of PC-SOD, the influence of PC-SOD on the antitumor activity of doxorubicin was investigated in vitro as well as in vivo. In vitro, it was found that PC-SOD, at concentrations that protected the heart against doxorubicin, did not interfere with the inhibition of cell proliferation induced by doxorubicin in three different cell types. Also in vivo, PC-SOD did not attenuate the doxorubicin-induced inhibition of xenograft growth in the nude mouse. It should be noted that PC-SOD protects the heart against a total dose of 24 mg·kg⁻¹ doxorubicin while completely preserving the antitumor activity of 16 mg·kg⁻¹ doxorubicin. The comparison of the protective efficacy of PC-SOD with the absence of an effect on the antitumor activity of doxorubicin may seem troublesome. The reasons that two different doses and dosing schedules of doxorubicin were used are simple. The cardioprotective efficacy of PC-SOD and the antitumor effect of doxorubicin are determined in two different strains of mice with different sensitivity toward doxorubicin. Doxorubicin at a cumulative dose of 16 mg·kg⁻¹ is maximally tolerated in the nude mice used to determine the antitumor effect of doxorubicin (Boven et al., 1990). The cardiotoxic effects of this dose of doxorubicin cannot be demonstrated by ECG measurements in BALB/c mice used to determine the cardioprotective efficacy of PC-SOD. Both models were developed independently of each other but are well validated and documented and therefore suitable to answer the question whether PC-SOD can serve as a cardioprotective compound during treatment with doxorubicin. However, an elegant extension of this study would be the determination of the cardioprotective effect of PC-SOD without an influence on the antitumor effect of doxorubicin in the same animal.

It can be concluded that pretreatment with PC-SOD ameliorates doxorubicin-induced cardiotoxicity. This find-

ing also provides further evidence that superoxide anions mediate the cardiotoxic effects of doxorubicin. The absence of an effect of PC-SOD on the antitumor activity of doxorubicin suggests that superoxide anions are not involved in the mechanism of the antitumor effect of doxorubicin, which is in line with the absence of such an effect in the cardioprotective flavonoid monoHER (van Acker et al., 1997). The antitumor effect of doxorubicin has been reported to depend mainly on the inhibition of topoisomerase II (Ravid et al., 1999).

Before the start of the present study, we speculated that high doses of PC-SOD might lead to toxic effects. The combination of hydrogen peroxide and a high SOD1 concentration could lead to peroxidative damage to bioorganic compounds and might thus increase damage at the site of oxygen radical formation (Kwon and Kang, 1999; Mao et al., 1993). However, even at the highest dose of PC-SOD that was used in this study ($80\,000\text{ U}\cdot\text{kg}^{-1}$), no reduction of cardioprotection or amplification of doxorubicin-induced cardiotoxicity was observed. Moreover, no signs of toxicity due to the high dose of PC-SOD were found, suggesting that PC-SOD has a large therapeutic window in the mouse.

In summary, it was shown that PC-SOD protects against doxorubicin-induced cardiotoxicity in the mouse when administered 15 min before doxorubicin. PC-SOD is well tolerated and shows no signs of toxicity even at very high doses. Very importantly, in our studies, PC-SOD did not show any sign of interference with the antitumor effect of doxorubicin. These data suggest that PC-SOD is a suitable candidate for further investigations on the prevention of doxorubicin-induced cardiotoxicity.

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